MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
7 February 2003

[redacted]
Sec. Research Graduate School Committee

FUNCTIONAL ROLES OF GROUP II METABOTROPIC GLUTAMATE RECEPTORS IN INJURY AND EPILEPSY

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

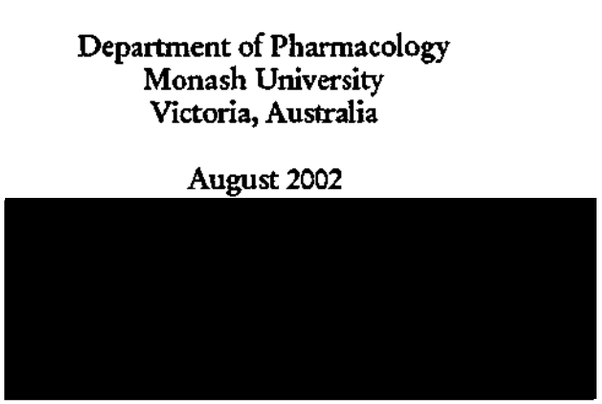
by

RANDAL XAVIER JOSEPH MOLDRICH

BSc (Hons) Monash University

Department of Pharmacology
Monash University
Victoria, Australia

August 2002



For Ronald Arthur King

and for those who suffer with him

TABLE OF CONTENTS

ACKNOWLEDGEMENT OF CONTRIBUTION TO THE RESEARCH

This thesis includes four original papers published in peer reviewed journals. The core theme of the thesis is the functional roles of group II metabotropic glutamate receptors in injury and epilepsy. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of Randal Xavier Joseph Moldrich (R.X. Moldrich), working within the Department of Pharmacology, Monash University under the supervision of Professor Philip M. Beart.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input via team-based research. In the following pages, details are given, by chapter, of the relative contributions of these authors to the research, and acknowledges the assistance of project funding and additional collaborators who were not authors.

Relative contributions of each author:

Chapter 2: $mGlu_{2/3}$ and in vitro injury

Paper 1: Moldrich, R.X., Giardina, S.F., Beart, P.M. (2001) Group II mGlu receptor agonists fail to protect against various neurotoxic insults induced in murine cortical, striatal and cerebellar granular pure neuronal cultures. *Neuropharmacology*, 41, 19–31.

Name:	% & nature of contribution:	Signature:
Moldrich, R.X.	70% All experiments, writing, ideas	[redacted]
Giardina, S.F.	10% Instruction of CGC culture	[redacted]
Beart, P.M.	20% Supervision, writing, ideas	[redacted]

Chapter 3: $mGlu_{2/3}$-mediated regulation of cAMP in neurons and astrocytes

Paper 2: Moldrich, R.X., Aprico, K., Diwakarla, S. O'Shea, R.D. Beart, P.M. (2002). Astrocyte $mGlu_{2/3}$-mediated cAMP potentiation is calcium sensitive: studies in murine neuronal and astrocyte cultures. *Neuropharmacology*. in press.

Name:	% & nature of contribution:	Signature:
Moldrich, R.X.	65% All experiments, writing, ideas	[redacted]
Aprico, K.	15% Development and generation of astrocytes	[redacted]
Diwakarla, S.	2.5% Technical assistance	[redacted]
O'Shea, R.D.	5% Development of astrocyte culture	[redacted]
Beart, P.M.	12.5% Supervision, writing	[redacted]

Chapter 4: $mGlu_{2/3}$ and epilepsy

Paper 3: Moldrich, R.X., Jeffrey, M., Talebi, A., Beart, P.M., Chapman, A.G., Meldrum, B.S. (2001) Anti-epileptic activity of group II metabotropic glutamate receptor agonists (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) and (−)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY389795). *Neuropharmacology*, 41, 8–18.

Name:	% & nature of contribution:	Signature:
Moldrich, R.X.	55% Most experiments, writing, ideas	[redacted]
Jeffrey, M.*	5% Kindling implantation	
Talebi, A.*	5% Some DBA sound-induced seizure experiments	
Beart, P.M.	5% Writing	
Chapman, A.G.	5% Development of techniques, writing	
Meldrum, B.S.	25% Development of techniques, writing, ideas	

Chapter 4: $mGlu_{2/3}$ and epilepsy

Paper 4: Moldrich, R.X., Talebi, A., Beart, P.M., Chapman, A.G., Meldrum, B.S. (2001) The $mGlu_{2/3}$ agonist, 2*R*,4*R*-aminopyrrolidine-2,4-dicarboxylate, is anti- and proconvulsant in DBA/2 mice. *Neuroscience Letters*, 299, 125-129.

Name:	% & nature of contribution:	Signature:
Moldrich, R.X.	65% Most experiments, writing, ideas	[redacted]
Talebi, A..*	5% Some DBA sound-induced seizure experiments	
Beart, P.M.	5% Writing	
Chapman, A.G.	5% Development of techniques, writing	
Meldrum, B.S.	20% Development of techniques, writing, ideas	

* Signed for on behalf of the Chief Investigator, B.S. Meldrum, since these authors were unable to be contacted.

Other specific acknowledgements:

The group II metabotropic glutamate receptor ligands, LY379268, LY389795 and LY307452 were used for only particular experiments in this thesis under agreement with Eli Lilly & Co. In some cases when either LY379268 or LY389795 were not available the next most selective agonists 2*R*,4*R*-APDC was purchased commercially.

Chapter 2:

This study was supported by an Australia Research Council Small Grant and in part by the National Health and Medical Research Council (Australia). The authors wish to acknowledge discussions with Dr. A.E. Kingston (Eli Lilly, IN, USA).

Chapter 3:

This study was supported by the National Health and Medical Research Council (Australia) of which P.M.B. is a Senior Principal Research Fellow, and the Rebecca L. Cooper Medical Research Foundation. The authors wish to acknowledge Yukiko Saihara for technical assistance, helpful discussions with Dr. B. Kemp and Dr. A.J. Lawrence (Dept. Pharmacology, Monash University), and the generous gift of LY379268 from Dr. D.D. Schoepp (Eli Lilly & Co., IN, USA).

Chapter 4:

Thankyou to the Epilepsy Research Foundation (UK) and the Fund for Epilepsy (UK) for providing financial support for this study. R. Moldrich was partly supported by the Centre for Neuroscience (Monash University) and the Pro-Vice Chancellor of Monash University, London. Special thanks to Dr. A.E. Kingston, Dr. J.A. Monn and Dr. J. Prather of Eli Lilly & Co. (Indiannapolis, IN, USA) for the generous gift of LY379268 and LY389795. Thanks finally to P. Yip for providing advice and assistance.

GENERAL ACKNOWLEDGEMENTS

Firstly, I'd like to thank Phil for his guidance throughout this doctorate, for his enthusiasm and love of French film and literature. Merci beaucoup. I particularly appreciate you sharing your insights into academia. It has meant as much to me as the science I have learnt along the way.

I want to thank Brian Meldrum and Astrid Chapman who helped to make my time at the Institute of Psychiatry in London very enjoyable and productive. Your ongoing friendship is a source of comfort and inspiration. At this point I would also like to thank Michele, Ping, Ellen, Maria and Ali for the many ways in which they helped me feel at home at the Institute.

In my lab, I want to thank Ross, Fiona, Nicole and Linda for their advice throughout my PhD and in the preparation of this thesis.

Thanks to those that inhabit the corridors of the Department of Pharmacology, especially Tony, Laima, Graeme, Dawn and Petra. Thanks also to Bevyn Jarrott and Roger Summers for always being willing to help out.

At many stages during my PhD, Michelle, David and Arthur at Medical Illustration have been kind to allow me to impose on them various posters and diagrams (some of which appear in this thesis) – thanks heaps.

There are many people with whom I've spent some enjoyable moments with over the last few of years. Unfortunately I can't thank you all. But I would like to thank in particular Lach, Jarrod, Stuart Mc., Dan, Song, Jezza and Ben for the many adventures within and outside the department – they have and will provide many great memories. I extend similar thanks to Sarah, Sharmaine, Eva, Courtney, Chrissandra, Emma and Dora. Special thanks, and best

wishes, to Karina who has almost been like a sister to me in the department. В благодарность за её доброту и дружбу я желаю Юлии любви и счастья.

I want to acknowledge the unconditional love and support shown by my mother, father and sister – particularly during my absence and in those uncertain times. In many ways my mum and dad have contributed as much as I, if not more, to the successful completion of this thesis. To them, I extend my congratulations.

To my mentor and best friend, Stuart: another stage in my life, and our friendship, has reached its conclusion. Thanks for being a mountain of unending support and inspiration. I look forward to spending many more fulfilling years with you.

Y para mi mimosa, Machű: through your generosity you have taught me that love is not about what you give, but what you give up. J'espere que ta vie est pleine de paz, d'amor y alegría. Salud! Gracias, cariños y besos.

PUBLICATIONS

COMMUNICATIONS ARISING FROM THIS WORK:

Refereed journal articles:

1. **Moldrich, R.X.**, Aprico, K., Diwakarla,S. O'Shea, R.D. Beart, P.M. (2002). Astrocyte $mGlu_{2/3}$-mediated cAMP potentiation is calcium sensitive: studies in murine neuronal and astrocyte cultures. *Neuropharmacology*. in press.

2. **Moldrich, R.X.**, Jeffrey, M., Talebi, A., Beart, P.M., Chapman, A.G., Meldrum, B.S. (2001) Anti-epileptic activity of group II metabotropic glutamate receptor agonists (–)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) and (–)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY389795). *Neuropharmacology*, 41, 8–18.

3. **Moldrich, R.X.**, Giardina, S.F., Beart, P.M. (2001) Group II mGlu receptor agonists fail to protect against various neurotoxic insults induced in murine cortical, striatal and cerebellar granular pure neuronal cultures. *Neuropharmacology*, 41, 19–31.

4. **Moldrich, R.X.**, Talebi, A., Beart, P.M., Chapman, A.G., Meldrum, B.S. (2001) The $mGlu_{2/3}$ agonist, 2*R*,4*R*-aminopyrrolidine-2,4-dicarboxylate, is anti- and proconvulsant in DBA/2 mice. *Neuroscience Letters*, 299, 125-129

Abstracts:

1. **Moldrich, R.X.**, Beart, P.M., (in press) The mGlu2/3 agonist induces adenosine release via phospholipase C and calmodulin kinase in cultured murine astrocytes. *Neuropharmacology*

2. Beart, P.M., **Moldrich, R.X.**, Aprico, K., Diwakarla, S., O'Shea, R.D., (in press). Astrocyte $mGlu_{2/3}$-mediated cAMP potentiation is calcium sensitive: studies in murine neuronal and astrocyte cultures. *Neuropharmacology.*

3. **Moldrich, R.X.**, Beart, P.M. (2002). Group II metabotropic glutamate receptor-mediated inhibition of cAMP is modulated by calcium and magnesium *in vitro. FENS Abstracts Vol.1*, A214.13.

4. **Moldrich, R.X.**, Beart, P.M. (2002) Cyclic AMP regulation in vitro by the group II metabotropic glutamate receptor ($mGlu_{2/3}$) agonist 2*R*,4*R*-aminopyrrolidine-2,4-dicarboxylate (APDC). *Proceedings of the Australian Neuroscience Society*, 13:197.

5. **Moldrich, R.X.**, Beart, P.M. (2001). The mGlu2/3 agonist 2*R*,4*R*-aminopyrrolidine-2,4-dicarboxylate (2*R*,4*R*-APDC) and injury in murine pure neuronal cultures. *Journal of Neurochemistry* 78(Suppl. 1):S190

6. **Moldrich, R.X.**, Beart, P.M., Meldrum, B.S. (2001) Anti-epileptic activity of group II metabotropic glutamate receptor ($mGlu_{2/3}$) agonists. *Proceedings of the Australian Neuroscience Society*, 12:38

7. **Moldrich, R.X.**, Beart, P.M. (2000) Group II metabotropic glutamate receptors and neurotoxicity in murine cortical, striatal and cerebellar granule cells. *Proceedings of the Australian Neuroscience Society*, 11:174

8. **Moldrich, R.X.**, Beart P.M. (1999) Agonists for type II mGluRs and neurotoxicity in murine cortical neuron cultures. *Neuropharmacology*, 38 (10):A31

COMMUNICATIONS ARISING FROM OTHER WORK:

Refereed journal articles:

1. Church, J.E., **Moldrich R.X.,** Beart, P.M., Hodgson, W.C. (submitted). The modulation of intracellular Ca^{2+} levels by Scorpaenidae venoms. *British Journal of Pharmacology.*

2. **Moldrich, R.X.,** Beart, P.M., Jane, D.E., Chapman, A.G., Meldrum, B.S. (2001) Anticonvulsant activity of 3,4-dicarboxyphenylglycines in DBA/2 mice. *Neuropharmacology*, 40, 732–735. (Appendix V)

3. **Moldrich, R.X.,** Beart, P.M., Pascoe, C.J., Cheung, N.S. (2000) Low-affinity kainate receptor agonists induce insult-dependent apoptosis and necrosis in cultured murine cortical neurons. *Journal of Neuroscience Research*, 59, 788-796.

4. **Moldrich, R.X.,** Cheung, N.S, Pascoe, C.J., Baker, S.R., Pedregal, C., Beart, P.M. (2000) Excitotoxic profile of LY339434, a GluR5 agonist, in cultured murine cortical neurons. *Brain Research*, 862, 270-275.

5. **Moldrich, R.X.,** Cheung, N.S., Pascoe, C.J., Beart, P.M. (1999) Excitotoxic injury profiles of low-affinity kainate receptor agonists in cortical neuronal cultures. *European Journal of Pharmacology*, 378, R1-R3.

Reviews, etc:

1. Giardina, S.F., Carroll, F.Y., **Moldrich, R.X.,** Finkelstein, D.I., Horne, M.K., Lawrence, A.J., Cheung, N.S., Beart, P.M. (2000) Kainate receptors, cellular signalling and excitotoxic neuronal death: new pharmacological evidence for the functional involvement of low-affinity and metabotropic kainate receptors. In *Excitatory Amino Acids: Ten Years Later*, (Eds) Cavalheiro et al. IOS Press.

Abstracts:

1. J.E. Church, **Moldrich, R.X.,** Beart, P.M., Hodgson, W.C. (2002) Stonefish (Synaceia trachynis) venom produces an increase in intracellular Ca^{2+} concentration in murine cortical neurons. *Proceedings of the Australian Neuroscience Society*, 13:132.

2. **Moldrich, R.X.,** Cheung, N.S., Beart, P.M. (1999) Low-affinity kainate receptors and excitotoxic neuronal death. *Proceedings of the Australian Neuroscience Society*, 10:155.

3. Beart, P.M., **Moldrich, R.X.,** Pascoe, C.J., Cheung, N.S. (1999) Excitotoxicity via low-affinity kainate receptors occurs across an insult-dependent, apoptotic-necrotic continuum. *Journal of Neurochemistry*, 73 (suppl. 1):S184

4. Beart, P.M., **Moldrich, R.X.,** Cheung, N.S. (1998) Low-affinity kainate receptors elicit excitotoxicity across an insult-dependent continuum involving apoptosis. *Proceedings of ASCEPT*, 5:44.

5. Beart, P.M., Carroll, F.Y., Giardina, S.F., John, C.A., **Moldrich, R.X.,** Pascoe, C.J., Cheung, N.S. (1998) Pharmacological and molecular analysis of iGluR-mediated apoptosis and necrosis. *Ionotropic Glutamate Receptors*. Pergamon Press, p.55.

SUMMARY

The thesis examines the function of group II metabotropic receptors for L-glutamate (Glu) in brain using a range drugs acting at these receptors under normal and pathological conditions, and employing experimental strategies allowing the investigation of neuronal injury *in vitro* and epilepsy *in vivo*.

Glu is the principal excitatory neurotransmitter of the central nervous system, and is involved in numerous physiological functions including learning and memory, and motor function. Disruption of Glu homeostasis is known to cause neurotoxicity and convulsive seizures, thereby contributing to the neuropathology of many neurological disorders including Alzheimer's disease, amyotrophic lateral sclerosis and epilepsy. Most of what is known regarding the role of Glu in these disorders comes from research with agonists and antagonists for the ligand-gated ion channels (NMDA, AMPA, KA). However, it has become evident that a more subtle, and indirect approach to the pharmacological manipulation of glutamatergic neurotransmission is required involving the newly developed ligands for the G protein-coupled metabotropic glutamate (mGlu) receptors (groups I-III).

Since group II mGlu receptors inhibit Glu release they are a potential target for the amelioration of neuronal injury. The ability of group II mGlu receptor agonists to attenuate injury induced by various insults was evaluated in cortical, striatal and cerebellar granular pure neuronal cultures. However, despite successful receptor coupling to intracellular signaling cascades, and regardless of culture development, agonist concentration, extent and mode of injury, group II mGlu receptor agonists were unable to protect against cellular death induced in these neuronal cultures. Shortly after this study, the role of trophic factor release from

astrocytes was demonstrated to mediate the neuroprotective actions of these group II mGlu receptor agonists.

Despite such evidence demonstrating the important role of astrocytes, signal transduction mechanisms of group II mGlu receptors remained a matter of some controversy. Therefore, new insights were sought into group II mGlu receptor function by studying cAMP production in cultured neurones and astrocytes, and by examining the inter-relationships of intracellular signalling to cellular calcium. Under physiological concentrations of Ca^{2+} and adenylate cyclase stimulation, an elevation of cAMP production was found, contrary to classical understanding of group II mGlu receptor function. This elevation of cAMP was mediated by phospholipase C- and calmodulin kinase II-dependent pathways, and results from the release of endogenous adenosine, which then acts at G_s protein-coupled A_{2A} receptors. Such mechanisms could influence the functional phenotypy of astrocytes under physiological and pathological conditions.

Finally, the ability of group II mGlu receptors to modulate excessive glutamatergic neurotransmission was investigated in animal models where this dysfunction is understood to contribute to the etiology of epilepsy. Sound-induced clonic seizures in DBA/2 mice were transiently inhibited by group II mGlu receptor agonists administered intracerebroventricularly or intraperitoneally (i.p.). The spike and wave discharge (SWD) duration of absence seizures in *lh/lh* mice was significantly reduced following infusion of the agonists while the electrically-induced seizure score and SWD of amygdala-kindled rats was partially inhibited following i.p. injection of the agonists. As a result of these experiments some group II mGlu receptor agonists were shown to be systemically active and potent anti-convulsants.

In conclusion, potent and selective group II mGlu receptor agonists possess neuroprotective activity via stimulation of astrocytes, which involves the release of trophic

factors and possibly adenosine. Group II mGlu receptor agonists showed anti-seizure activity in rodent models of human epilepsy and are understood to effect this outcome by inhibiting Glu release from presynaptic glutamatergic terminals. Such agonists may prove useful in the treatment of human neurological disorders that involve degenerative mechanisms and epilepsy.

ABBREVIATIONS

ABH x D-I	(1*S*,2*S*,4*S*,5*S*)-2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid
AC	adenylate cyclase (or adenylyl cyclase)
ACh	acetylcholine
1*S*,3*R*-ACPD	1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid
AD	Alzheimer's disease
trans-ADA	*trans*-azetidine-2,4-dicarboxylic acid
ADD	after discharge duration
AED	anti-epileptic drug
AIDA	(*RS*)-1-aminoindan-1,5-dicarboxylic acid
ALS	amyotrophic lateral sclerosis
Am	amygdala
AMP	adenosine monophosphate
AMPA	(*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
trans-APDC	*trans*-4-aminopyrrolidine-2,4-dicarboxylic acid
1*S*,3*R*-APDC	1*S*,3*R*-4-aminopyrrolidine-2,4-dicarboxylic acid
1*S*,3*S*-APDC	1*S*,3*S*-4-aminopyrrolidine-2,4-dicarboxylic acid
2*R*,4*R*-APDC	2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylic acid
L-AP3	*S*-3-phosphono-2-aminobutyric acid
L-AP4	*S*-4-phosphono-2-aminobutyric acid
Arg	arginine
ATP	adenosine triphosphate
BAY36-7620	(3a*S*,6a*S*)-6a-naphtalen-2-ylmethyl-5-methylidenhexahydro-cyclopental[c]furan-1-on
BDNF	brain-derived neurotrophic factor
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
$[Ca^{2+}]_e$	extracellular Ca^{2+} concentration
CaM	calmodulin
CaMK	calmodulin kinase
cAMP	cyclic adenosine monophosphate
L-CBG-I	L-(2*S*,1'*S*,2'*S*)-2-(2-carboxycyclobutyl)glycine
L-CCG-I	L-(2*S*,1*S*,2'*S*)-2-carboxycyclopropylglycine
CGC	cerebellar granule cell
CHPG	2-chloro-5-hydroxyphenylglycine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPCCOEt	7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester
4CPG	*S*-4-carboxyphenylglycine
4C3HPG	*S*-4-carboxy-3-hydroxyphenylglycine
DAG	diacylglycerol
DBA/2	dilute brown agouti (mice)
DCG-IV	(2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine
DCPG	3,4-dicarboxyphenylglycine
DHPG	*S*-3,5-dihydroxyphenylglycine
DHPMP	amino-3,5-dihydroxyphenylmethylphosphinic acid
div	days *in vitro*
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate

DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
EAA	excitatory amino acid
EAAC1	excitatory amino acid carrier 1 (human: EAAT3)
EAAC4	excitatory amino acid carrier 4 (human: EAAT4)
EAAT	excitatory amino acid transporter (1-5)
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EGlu	*S*-α-ethylglutamic acid
Epac	exchange protein activated by cAMP
ERK1/2	extracellular signal-regulated kinase 1/2
L-F2CCG-I	L-(2*S*,1'*S*,2'*S*)-3',3'-difluoro-2-(carboxycyclopropyl)glycine
GABA	γ-aminobutyric acid
GEP	genetically epilepsy prone-9 (rats)
GFAP	glial fibrillary acidic protein
GIRK	G protein-coupled inwardly rectifying K^+ channel
GLAST	glutamate/L-aspartate transporter (human: EAAT1)
Gln	L-glutamine
GLT1	Glu transporter 1 (human: EAAT2)
Glu	L-glutamate
GluT	glutamate transporter
GPCR	G protein-coupled receptor
GST	general seizure threshold
GTP	guanine triphosphate
GYKI 52466	1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3benzodiazepine
H89	*N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide
3-HPG	*S*-3-hydroxyphenylglycine
5HT	serotonin
IBMX	3-isobutyl-1-methylxanthine
IBO	ibotenate
ic or i.c.	intracollicular
icv or i.c.v.	intracerebroventricular
iGlu	ionotropic glutamate (receptor)
i.p.	intraperitoneally
IP	inositol 5-phosphate
IP_3	inositol 1,4,5-triphosphate
ISO	isoprenaline
KA	kainate
KN-62	(*S*)-isoquinolinesulphonic acid ester
lh/lh	lethargic (mice)
LTD	long-term depression
LTP	long-term potentiation
LY341495	2*S*-2-amino-2-(1*S*,2*S*-2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoic acid
LY354740	(1*S*,2*S*,5*R*,6*S*)-(+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
LY379268	(–)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY389795	(–)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
MAP-2	microtubule associated protein-2
MAP4	*S*-α-methyl-2-amino-4-phosphonobutanoic acid

MAPK	mitogen-activated protein kinase
MCCG	(2*S*,1'*S*,2'*S*)-2-methyl-2-(2-carboxycyclopropyl)glycine
t-MCG-I	*trans*- (2*S*,1'*S*,2'*R*,3'*S*)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine
cis-MCG-I	(2*S*,1'*S*,2'*R*,3'*R*)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine
MCPA	*S*-α-methyl-3-carboxyphenylalanine
MCPG	*S*-α-methylcarboxyphenylglycine
S-4-MeG	*S*-4-methyleneglutamic acid
2*S*,4*S*-4-MG	(2*S*,4*S*)-4-methlyglutamate
mGlu	metabotropic glutamate (receptor)
MK-801	(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine maleate (or dizoclipine)
MPEP	2-methyl-6-(phenylethynl)pyridine
MPP^+	1-methyl-4-phenylpyridinium
MPPG	(*R*,*S*)-α-methyl-4-phosphonophenylglycine
mRNA	messenger ribonucleic acid
MSOP	(*RS*)-α-methylserine-*O*-phosphate
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
NAAG	*N*-acetylaspartylglutamate
NAc	nucleus accumbens
NECA	5'-*N*-ethyl-carboxamidoadenosine
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NR	NMDA receptor subunit
NTS	nucleus tractus solitarii
PAG	periaqueductal gray
PDE	phosphodiesterase
PDZ	PSD-95 disc-large zona occludens 1
PIC	picrotoxin
PICK	protein-interacting c-kinase-I
PI3K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA/B/C	protein kinase A/B/C
PLC/D	phospholipase C/D
(*RS*)-PPG	(*R*,*S*)-4-phosphonophenylglycine
PSD-95	post-synaptic density protein-95
PTX	pertusis toxin
PTZ	pentylenetetrazol
Quis	Quisqualate
RNA	ribonucleic acid
Ser	serine
SN	substantia nigra
L-SOP	L-serine-O-phosphate
SWD	spike wave discharge
TCA	tricarboxylic acid cycle
TGF	transforming growth factor
Thr	threonine
TMD	transmembrane domain

TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin end labelling
U73122	(1-[6-([(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione
UBP1111	α-methyl-3-chloro-4-phosphonophenylglycine
ZM241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

CHAPTER 1

GENERAL INTRODUCTION

1.1 L-GLUTAMATE AND THE CENTRAL NERVOUS SYSTEM

1.1.1 L-Glutamate

L-Glutamate (Glu) or L-glutamic acid is an amino acid that was discovered nearly fifty years ago to have excitatory actions on neurones of the central nervous system (CNS) (Hayashi, 1954; Curtis and Watkins, 1960). Although identified as a neuroexcitant, Glu was only considered important as a non-essential amino acid in nitrogen metabolism (Curtis and Johnston, 1974; Erecinska and Silver, 1990). Furthermore, Glu mediates various biochemical functions, including fatty acid and protein synthesis, energy metabolism, and is a precursor for various endogenous small molecules such as folic acid, and for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Erecinska and Silver, 1990). Despite being present in high concentrations and exhibiting an ubiquitous distribution throughout the brain, only some twenty years later was it accepted that Glu satisfied the four main criteria for classification as a neurotransmitter (Fonnum, 1991): (1) presynaptic localisation, (2) release by physiological stimuli (including vesicular storage (Storm-Mathisen et al., 1995)), (3) identical action with a known, naturally occurring transmitter, and (4) association with a mechanism for rapid termination of transmitter action (e.g. Glu uptake systems; Balcar and Johnston, 1972). Now, Glu is understood to be the principal excitatory amino acid (EAA) neurotransmitter in the mammalian CNS where it plays roles in numerous physiological functions including synaptic plasticity, learning and memory, sensation (Broman et al., 2000) and cardiovascular regulation (Collingridge and Singer, 1990).

1.1.2 Glutamate Synthesis and Metabolism

Glu is a non-essential amino acid that is readily synthesised by the body. Under physiological conditions Glu is not able to cross the blood-brain barrier, and therefore plasma

levels do not reflect those levels found in the CNS (Fonnum, 1984). Glu that is required for neurological functions is synthesised within the CNS (Fonnum, 1984) and remains at a relatively constant concentration (Erecinska and Silver, 1990). In general, Glu concentrations in the CNS measure around 1 μM in the extracellular fluid and up to 10,000-fold higher in the cytosol (Schousboe, 1981; Fonnum, 1984), and up to 100 mM in the vesicles of nerve terminals (Burger et al., 1989; Shupliakov et al., 1992).

While Glu synthesis can occur through various pathways, it seems that Glu is mainly synthesised from glucose by the process of glycolysis such that glucose is the most important precursor of Glu (Minchin and Beart, 1975). Glucose enters the brain by crossing the blood-brain barrier and is taken up into astrocytes via glucose transporters. Here, glucose is broken down to pyruvate, which in turn enters the tricarboxylic acid cycle (TCA) where finally Glu is produced from α-ketoglutarate (see Figure 1.1). L-Glutamine (Gln) too can be converted to Glu by phospho-activated glutaminase, an action that largely depends upon glial cells (such as astrocytes). Glu is also taken up by glial cells and converted to Gln, in an adenosine triphosphate (ATP)-dependent manner, which is released into the extracellular space and rapidly taken up by neurones. Neurones then convert the Gln to Glu in the synaptic terminals (Erecinska and Silver, 1990; Nicholls and Attwell, 1990) and Glu is transported into vesicles for storage (Naito and Ueda, 1985). Glu release is believed to be via a classical exocytotic, calcium-dependent pathway utilising synaptic vesicles and requiring membrane depolarisation (Nicholls and Attwell, 1990). Figure 1.1 outlines some of the key metabolic processes involved in the biochemistry of Glu.

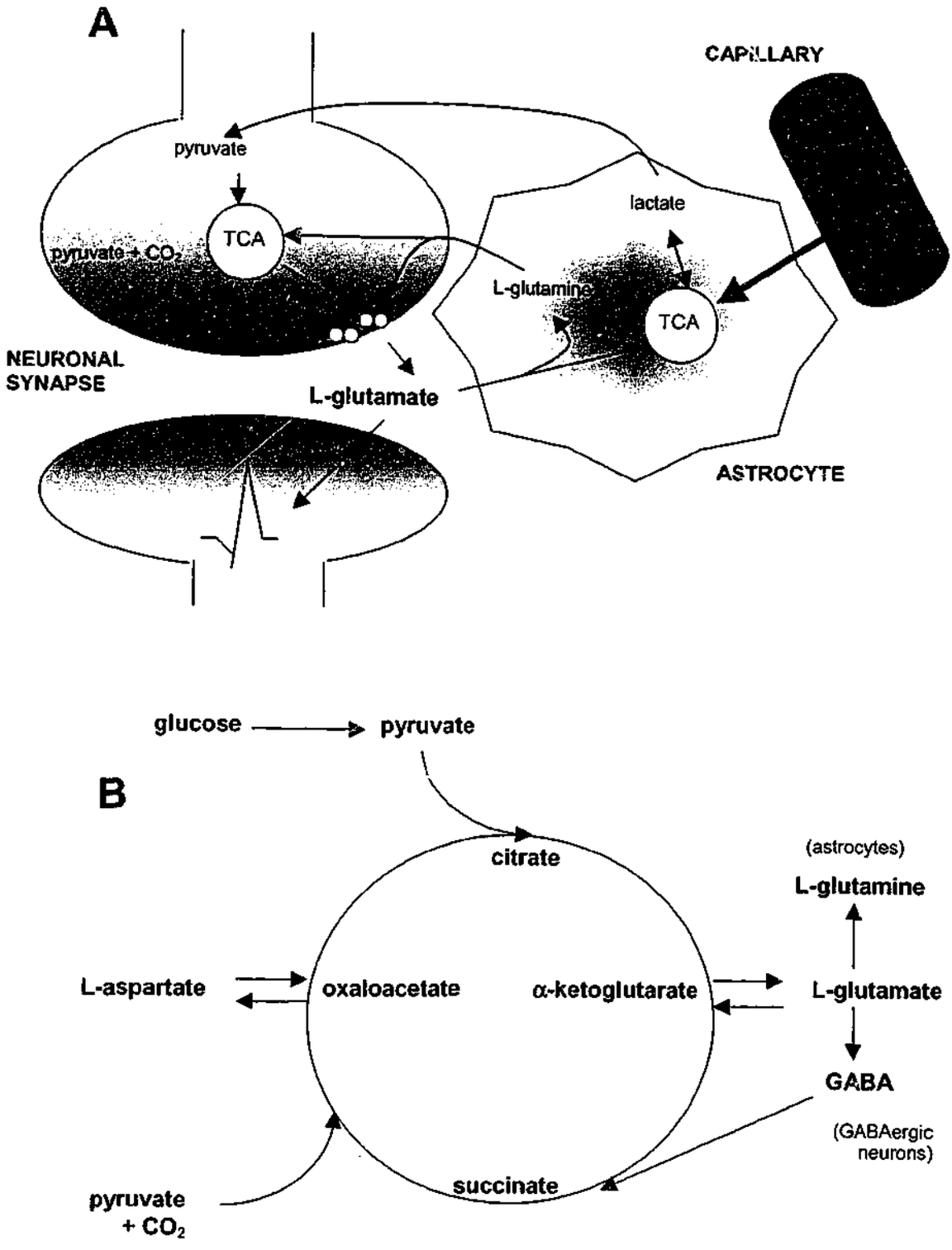


Figure 1.1 Schematic representation of key pathways in Glu synthesis and metabolism: between neurons and glia (A) and involving the tricarboxylic acid cycle (TCA) (B).

1.1.3 Glutamate Uptake Systems

Early investigations with the use of various enzyme inhibitors had failed to provide evidence for the augmentation of excitatory responses to EAAs, implying that Glu was not degraded after release (Curtis and Watkins, 1960). However, Curtis and colleagues later demonstrated that Glu responses were enhanced by the addition of the membrane transport inhibitor p-chloromercuriphenylsulphonate in feline Renshaw cells (McCulloch et al., 1974). Since then a family of Glu transporters (GluTs) has since been cloned and characterised pharmacologically (Vandenberg, 1998; Danbolt, 2001).

Once Glu is released from nerve terminals it is quickly removed from the extracellular space by GluTs. At present five Glu transporters have been characterised in rodent brain: the Glu/L-aspartate transporter (GLAST), Glu transporter 1 (GLT1), excitatory amino acid carrier 1 (EAAC1) and excitatory amino acid carrier 4 (EAAC4) and excitatory amino acid transporter 5 (EAAT5) (Vandenberg, 1998). In the human brain five GluT homologues have been cloned and are termed excitatory amino acid transporter 1-5 (EAAT1-5) (Sims and Robinson, 1999). Other members and splice variants of this family are likely to be discovered as the transporters cloned thus far are predominantly localised on glia or postsynaptic neurones, and early evidence suggests that some component of Glu transport occurs into presynaptic nerve terminals (Beart, 1976). For example, recently, glial GluT splice variants (GLT1b and GLT1v) has been reported to be localised to nerve terminals (Chen et al., 2002; Schmitt et al., 2002). Table 1.1 provides an overview of the tissue distribution, cellular localisation and the relative affinities of the transporters for Glu.

As Glu is a potent neurotoxin (Meldrum and Garthwaite, 1990), rapid removal of Glu from the extracellular space is required to avoid neuronal damage, so much so that various disease states may be linked to abnormal functioning of various GluTs (Vandenberg, 1998;

Table 1.1 Characteristics of Glu transporters

Glu Transporter	GLAST (EAAT1)	GLT1 (EAAT2)	EAAC1 (EAAT3)	EAAC4 (EAAT4)	EAAT5
CNS Cellular Localisation	Glia	Glia	Neurons	Neurons	Neurons
Tissue Distribution	Brain Heart Lung Placenta Skeletal muscle	Brain Liver	Brain Intestine Kidney Liver Heart	Cerebellum Hippocampus Placenta	Retina
Affinity for Glu (Km)	18-20 μM	18 μM	30 μM	2.5 μM	64 μM

Where different, human Glu transporter names appear in brackets.
(Adapted from Kanai et al., 1997; Vandenberg, 1998; Pow and Barnett, 2000).

Danbolt, 2001). For example, while significant changes in GLAST or GLT1 expression are not evident in post-mortem Alzheimer's disease brain when compared to aged matched controls, uptake studies have demonstrated a decrease in GLT1 activity in Alzheimer's patients by approximately 34% (Scott et al., 1995; Masliah et al., 1996). There is also evidence to suggest that in post-mortem brains of Alzheimer's patients some GluTs are differentially regulated due to a lower ratio of N-terminal immunoreactivity when compared to that of central immunoreactivity of GLT1 (Beckstrom et al., 1999). The human glial transporter EAAT2 is down-regulated by up to 95% in the motor cortex and spinal cord of patients suffering amyotrophic lateral sclerosis, possibly due to aberrant RNA splicing (Lin et al., 1998). Studies employing antisense oligonucleotides and the production of knockout mice have demonstrated the importance of GLAST, GLT1 and EAAC1 in synaptic transmission since these animals develop seizures (reviewed in: Meldrum et al., 1999). Therefore, atypical functioning of Glu uptake mechanisms may be a common attribute in neurodegenerative diseases, whereby excess Glu remains in the extracellular space and receptor overstimulation causes neuronal injury.

1.1.4 Glutamatergic Pathways

Until recently, identification of pathways that utilise Glu as a neurotransmitter has been difficult since Glu is also a metabolic intermediate (as described in section 1.1.2), consequently immunohistochemistry could not always be correlated with neurotransmitter levels. Identification of glutamatergic pathways finally came from studies involving high affinity uptake, selective lesioning of neurones, monitoring endogenous release and retrograde transport (Fagg and Foster, 1983; Fonnum et al., 1981). More recently glutamatergic neurones have been mapped by retrograde transport of [^{3}H]-D-aspartate (Beart et al., 1994) and by immunocytochemistry with Glu antibodies (Storm-Mathisen et al., 1995). Numerous pathways

employ Glu as their neurotransmitter, including those originating from neocortical pyramidal cells, several intrahippocampal pathways and parallel fibres of the cerebellum (Feldman et al, 1997; Fonnum, 1984; Storm-Mathisen et al., 1995). Glutamatergic efferents from various areas of the neocortex project to many regions of the brain including to the nucleus accumbens (Walaas, 1981), the amygdala and thalamus (Walker and Fonnum, 1983). In one of the few studies combining neurochemical mapping and *in vivo* electrophysiology, a glutamatergic pathway was found to link the ventromedial hypothalamus to the periaqueductal gray (Christie et al., 1985; Christie et al., 1986). Efferent pathways also exist that originate in the cerebellum and project to the inferior olive, while within the cerebellum parallel fibres arise from the granule cells (Storm-Mathisen et al., 1987).

In the basal ganglia circuitry, glutamatergic afferents from the cortex project to medium spiny GABAergic neurones of the striatum (Bellomo et al., 1998) and the subthalamic nucleus, and efferents innervate the cortex from the thalamus. These pathways are often exploited to induce animal models of Parkinson's disease or to prevent Parkinson-like symptoms thought to arise from the basal ganglia circuitry.

Within the hippocampus, granule cells of the dentate gyrus send axons (mossy fibres) which terminate on pyramidal neurones in the CA3 region. These neurones in turn project to the fibre tract, the fornix, which is a major efferent of the hippocampus to the septal area, while collateral branches (Schaffer collaterals) project from the CA3 to the CA1. Many of these pathways have been shown to be involved in Glu receptor-mediated long-term potentiation (Bortolotto et al., 1999).

1.2 GLUTAMATE RECEPTORS

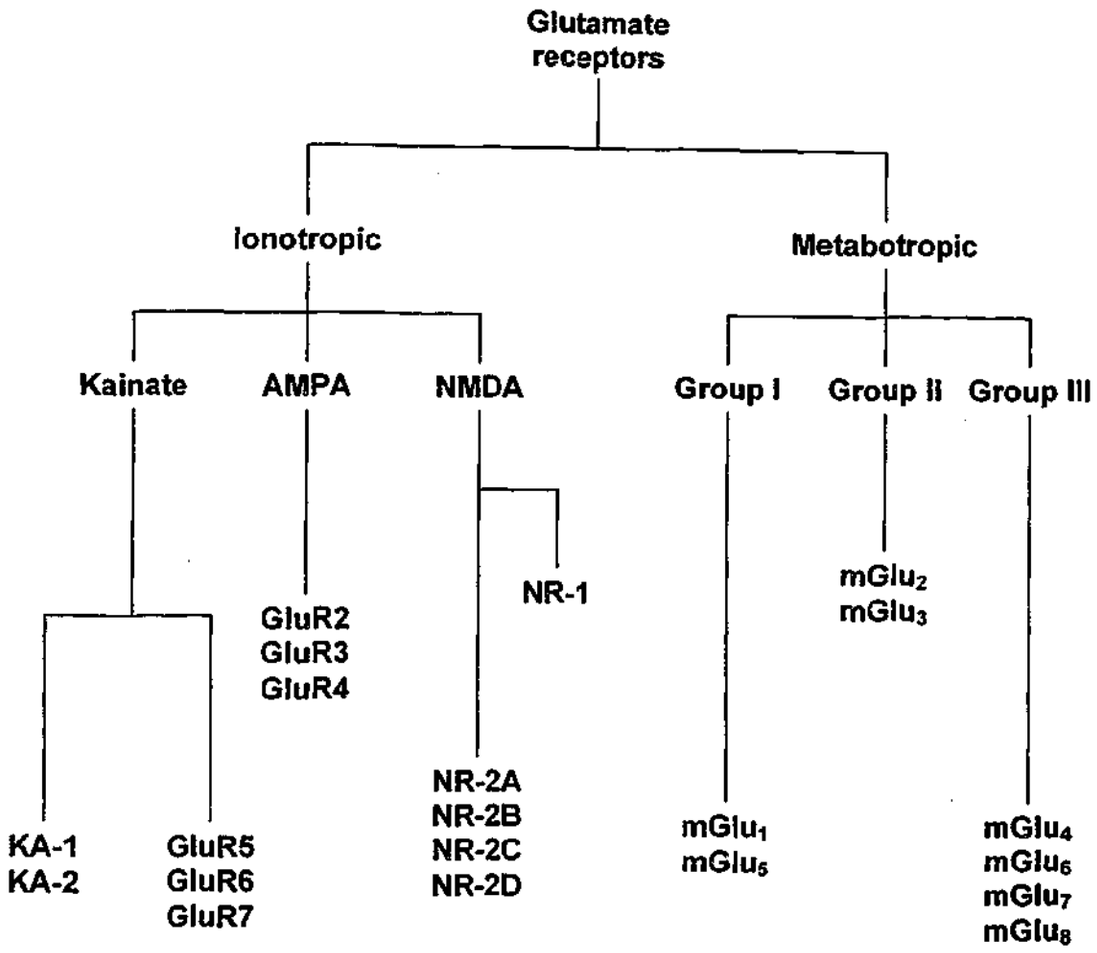
1.2.1 History of glutamate receptors

Since neurones were almost universally excited by Glu, receptors for Glu were thought to be widely distributed throughout the brain and spinal cord (Hayashi, 1954). Later, potency differences were found across various brain regions which suggested the presence of different subtypes of Glu receptor (Duggan, 1974). Initially, receptor subtypes were classified by their differential sensitivities to EAAs, with greater definition coming from early antagonists. Finally, through cloning, sequencing and expression a fuller understanding of the differences in Glu receptor subtypes has emerged.

1.2.2 Classification of glutamate receptors

Mammalian CNS Glu receptors fall into two broad categories, chiefly distinguished by their mechanism of action: ionotropic (iGlu) and metabotropic (mGlu) Glu receptors. Ionotropic Glu receptors are pentomeric ligand-gated ion channels of four transmembrane domains (TMD) (with the exception of NMDA receptors which have two re-entrant domains; Bettler and Mulle, 1995; Dingledine et al., 1999). Activation of iGlu receptors initiates a cation influx and results in a rapid membrane depolarisation. These receptors can be subdivided into three receptor groups based on their activation by the agonists *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (KA). Each of these three groups consist of distinct receptor subunits that assemble in homomeric or heteromeric complexes. Unlike iGlu receptors, mGlu receptors activate signal transduction mechanisms through G proteins coupled to the intracellular terminus of the receptor (mGlu receptors are discussed in more detail later). Figure 1.2 demonstrates the relationship between the family of Glu receptors.

Figure 1.2 Cloned glutamate receptors.



Phylogenic tree demonstrating the relationship between the Glu receptors and their subunits. The nomeclature of iGlu receptors is currently under review.

1.2.3 iGlu receptors

Pharmacological advances have preceded the molecular biology of iGlu receptors and in the 1970s and 1980s they were simply classified as NMDA and non-NMDA receptors. With the knowledge that iGlu receptors were likely to mediate neuronal injury in many acute and chronic neurological conditions (Lipton and Rosenberg, 1994; Meldrum and Garthwaite, 1990), there have been many developments in the medicinal chemistry and structure-activity relationships of NMDA receptor agonists and antagonists (Rogawski, 1993; Speliotes et al., 1994; Small and Buchan, 1997). Subsequently in trials, many competitive and non-competitive NMDA receptor antagonists were found to have psychomimetic side effects and/or caused morphological changes in the brain (Olney et al., 1990; Schmutz et al., 1997). Interest in iGlu receptors as therapeutic targets for the management of neuronal injury continues, but has concentrated more recently on non-NMDA receptors (namely, AMPA and KA receptors). A small number of research groups continue to investigate the possible clinical roles of slow-channel NMDA receptor blockers (Monyer et al., 1994; Reith and Sillar, 1998; Vicini and Rumbaugh, 2000) and glycine-NMDA receptor antagonists (Sareen, 2002; Chenard and Menniti, 1999). Regardless, the pharmacological industry has long recognised the large financial gain that could be achieved by successful management of neurodegenerative conditions through the glutamatergic system, and due to the limited success of targeting iGlu receptors, much attention now focuses on mGlu receptors.

1.2.4 mGlu receptors

Metabotropic Glu receptors are guanine nucleotide or GTP-binding (G protein)-coupled receptors (GPCR), and have a monomeric structure consisting of seven TMDs. They belong to family 3 GPCRs, along with $GABA_B$, pheromone and Ca^{2+}-sensing receptors

(Bockaert and Pin, 1999), since they share little sequence similarity with the family of rhodopsin-like GPCRs (referred to as family 1) and large-peptide GPCRs (e.g. vasoactive intestinal polypeptide receptors; family 2). G proteins consist of three subunits α, β and γ (Selbie and Hill, 1998; Antoni, 2000). The α subunit is generally responsible for activating the second messenger associated with the relative mGlu receptor (i.e. PLC or AC). The β and γ subunits generally act as a dimer ($\beta\gamma$) although their exact function is unclear at present. Emerging roles for the $\beta\gamma$ subunit will be discussed in more detail in Chapter 3.

Metabotropic Glu receptors have been classified into three groups (I, II and III) based on the signal transduction pathway to which they couple (Table 1.2), amino acid sequence homology (Table 1.3) and agonist pharmacology (section 1.5). These signal transduction pathways can include activation of phospholipase C (PLC), adenylate cyclase (AC) and phospholipase D (PLD), and result in the regulation of protein and enzyme activity, and calcium homeostasis.

Group I mGlu receptors, which consist of splice variants of the subtypes $mGlu_1$ and $mGlu_5$, are generally made up of about 1100 amino acids and share approximately 60% homology within the group, and approximately 40% homology with groups II and III (Stephan et al., 1996). Group II mGlu receptors consist of $mGlu_2$ and $mGlu_3$ subtypes which share a 66% homology: they are made up of approximately 870 amino acids and are unique in that no splice variants have yet been identified (Flor et al., 1995a; Makoff et al., 1996a; Emile et al., 1996). Group III mGlu receptors range from 850 – 970 amino acids, consist of the subtypes $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$, and share 65 – 70% amino acid homology (Flor et al., 1995b; Makoff et al., 1996b,c; Wu et al., 1998). In general, rodent mGlu receptors share approximately 96% homology with their human counterparts (Emile et al., 1996; Hashimoto et al., 1997; Wu et al., 1998).

Table 1.2 Classification and transduction mechanisms of mGlu receptors in rat. Similar splice variants exist for human mGlu receptors.

Group	Subtypes	Splice Variants	Transduction
I	$mGlu_1$	a, b	↑ PLC, ↑ Ca^{2+}, ↓ K^+, ↓ VOCC, ↑ L-VOCC
	$mGlu_5$	a, b, c, e	
II	$mGlu_3$		↓ AC, ↓ VOCC
	$mGlu_2$		
III	$mGlu_4$	a, b	↓ AC, ↓ VOCC, ↑ K^+
	$mGlu_6$	a, b	
	$mGlu_7$	a, b	
	$mGlu_8$		
	mGlu?	?	↑ PLD

Key to symbols: ↑, stimulation; ↓, inhibition; AC, adenylate cyclase; Ca^{2+}, intracellular calcium concentration; K+, intracellular potassium concentration; VOCC, voltage operated calcium channels; L-VOCC, L-type voltage operated calcium channels; PLC/D, phospholipase C/D. (Pin et al., 1999)

Table 1.3 Percentage amino acid homology between mGlu receptors in rat.

mGlu	2	3	4	5	6	7	8
1	43.1	41.2	39.0	60.8	40.3	38.1	~43*
2		66.9	45.3	42.5	46.0	44.0	~46*
3			44.1	41.5	44.8	43.1	~46*
4				39.8	68.6	67.4	70
5					39.3	37.8	~43*
6						64.3	70
7							74

* Approximate values based on $mGlu_8$ homology to the mGlu receptor group. (Duvoisin et al., 1995)

Group I mGlu receptors are coupled to a stimulatory G protein (G_q) and activate inositol phosphate (IP) hydrolysis. G protein activation of PLC will generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-biphosphate (PIP_2). IP_3 then activates protein kinase C (PKC). Inositol phosphates are known to regulate membrane trafficking, glucose metabolism, cytoskeletal organisation, and most importantly, regulation of intracellular Ca^{2+} homeostasis – particularly the release of stored Ca^{2+} via IP_3-sensitive receptors (Chavis et al., 1995; Chavis et al., 1996). Secondary activity induced by these pathways includes activation of plasma membrane voltage operated Ca^{2+} channels (VOCC) and the induction of K^+ efflux via Ca^{2+}-sensitive K^+ channels (Fagni et al., 2000; Table 1.2).

Group II and III mGlu receptors act via an inhibitory G protein (G_i) to inhibit AC activity and prevent the formation of cyclic adenosine monophosphate (cAMP), which can result in the inhibition of VOCCs (Table 1.2). Ontogenetic and pharmacological studies show that some mGlu receptor agonists activate PLD (e.g. *t*-ADA), however debate exists over which group such receptors belong to (Pellegrino-Giampietro et al., 1996; Klein et al., 1997a,b).

Cyclic AMP is synthesised from ATP by AC and is broken down by phosphodiesterases (PDEs). Different AC isozymes mediate different cAMP responses according to the input given, and moreover, the same isozyme can produce opposite cAMP responses. In general, $G_{s\alpha}$ proteins stimulate AC production of cAMP, while G_i proteins inhibit production of cAMP (see reviews: Tesmer and Sprang, 1998; Antoni, 2000). However, the activity of AC is regulated by a number of factors including Ca^{2+}, calmodulin, calcineurin and $G_{\beta\gamma}$ proteins. For example, $G_{s\alpha}$ protein activation of AC isozyme I (AC I) generally stimulates cAMP production in neurones, thereby activating PKC, however a $G_{\beta\gamma}$ protein

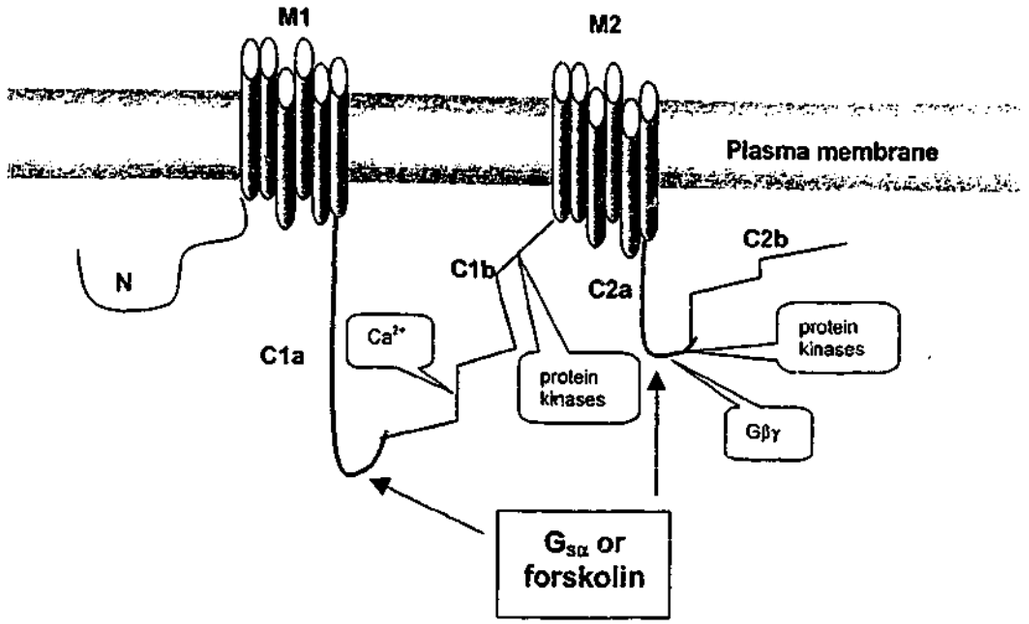
input (from either $G_{s\alpha}$ or $G_{i\alpha}$) could inhibit AC I. Isozymes V and VI are sensitive to Ca^{2+}, which inhibits the inhibitory or stimulatory response of these isozymes to $G_{i\alpha}$ or $G_{s\alpha}$, respectively. Figure 1.3 and Table 1.4 describe some of the regulatory components of the AC catalytic process.

Protein kinase A (PKA) is the classical intracellular target of cAMP. Upon binding of cAMP to the R subunit of PKA the C subunit dissociates and phosphorylates it substrates, which include nuclear proteins, enzymes and transcription factors (Taylor et al., 1990). Another key target of cAMP are cyclic nucleotide gated ion channels, which control the flux of Ca^{2+}, Na^{+} and K^{+}. Activation of the latter two channel types is thought to be responsible for the depolarising effects mediated by GPCRs associated with cAMP signalling (Tesmer and Sprang, 1998; Antoni, 2000). Another, novel group of cAMP targets include guanine triphosphate (GTP) exchange proteins. Recently it was found that activation of the small GTPase, Rap1, was enhanced by an 'exchange protein activated by cAMP' (Epac) which leads to protein kinase B activation – a key mediator of cell survival and differentiation (Mei et al., 2002).

1.3 mGlu RECEPTOR STRUCTURE AND FUNCTION

Glutamate was first thought to act via three EAA receptors: NMDA, KA and Quisqualate (Quis) receptors. Through such receptors, Glu had been shown to cause an influx of Na^{+} and Ca^{2+} to produce excitatory postsynaptic potentials (EPSP) – required for neuronal action potentials (Dudel, 1975; Kimura et al., 1985a,b; Mori-Okamoto and Ikeda, 1986). The formation of inositol phosphates had also been reported to be associated with Glu activity, and their formation in striatal neurones was shown to be induced according to the following order of potency: Quis > Glu > NMDA > KA (Sladeczek et al., 1985; Nicoletti et al., 1986).

Figure 1.3 Schematic representation of adenylate cyclase showing points of regulation.



Adenylate cyclase (AC) consists of an N-terminal region (N), two transmembrane domains (M1, M2) and catalytic domains (C1a, C1b, C2a, C2b). Sites of AC regulation are indicated by the callouts. Activation of AC by $G_{s\alpha}$ or forskolin induces the closing of C1a and C2a domains to form the catalytic core for the ATP binding site. Adapted from Antoni, 2000.

Table 1.4 Regulation of adenylate cyclase (AC) isozyme activity in the mammalian CNS by calcium, G proteins and protein kinases.

AC isozyme	G protein			Ca^{2+}	protein kinase
I	↑ $G_{s\alpha}$		↓ $G_{\beta\gamma}$	↑ CaM[a]	↑ PKC, ↓ CaMK-IV
II	↑ $G_{s\alpha}$	↓ $G_{i\alpha}$	↑ $G_{\beta\gamma}$?		↑ PKC
III	↑ $G_{s\alpha}$				↑ PKC, ↓ CaMK-II
IV	↑ $G_{s\alpha}$		↑ $G_{\beta\gamma}$?		↓ PKC
V	↑ $G_{s\alpha}$	↓ $G_{i\alpha}$		↓ (≤ 1 μM)	↑ PKC, ↓ PKA
VI	↑ $G_{s\alpha}$	↓ $G_{i\alpha}$		↓ (≥ 1 μM)	↓ PKC & PKA
VII	↑ $G_{s\alpha}$				↑ PKC
VIII	↑ $G_{s\alpha}$?			↑ CaM[a]	?
IX	↑ $G_{s\alpha}$	↓ $G_{i\alpha}$?		↓ calcineurin[a]	?

Key to symbols: ↑, stimulation of AC activity; ↓, inhibition of AC activity; CaM, calmodulin; CaMK, calmodulin kinase; PKC/A, protein kinase C/A; ?, activity unknown or indefinite.
[a] Stimulation by CaM or calcineurin is entirely dependent on Ca^{2+}.
Adapted from Antoni, 2000.

Following this discovery, further work showed that the Quis-induced response was mediated via PLC through the group I mGlu receptor (Recasens et al., 1987; Manzoni et al., 1990).

Around this time, the compound L-AP4 was shown to inhibit excitatory inputs *in vitro* (Hearn et al., 1986; Cotman et al., 1986), and its action was insensitive to NMDA receptor antagonists (Sheardown, 1988) and distinguishable from Quis responses (Whittemore and Koerner, 1989a). Later, it was found that L-AP4 acted to inhibit Glu release (Jones and Roberts, 1990; Adamson et al., 1990; Forsythe and Clements, 1990) and is now known to be a group III mGlu receptor agonist (Pook et al., 1993; Akazawa et al., 1994). In other studies, the regional content of the endogenous, putative neurotransmitter NAAG had been described in rat brain (Koller et al., 1984; Anderson et al., 1986; Forloni et al., 1987), and its rudimentary pharmacology mapped (Westbrook et al., 1986; Mori-Okamoto et al., 1987; Joels et al., 1987; Sekiguchi et al., 1987; Whittemore and Koerner, 1989b). Amongst mGlu receptors, NAAG is now regarded as a selective agonist of $mGlu_3$ (Wroblewska et al., 1997).

Metabotropic Glu receptors have a large extracellular domain of approximately 560 amino acids, which includes the amino (N)-terminus, while their seven TMDs are linked by three short intracellular loops and three extracellular loops. Highly conserved regions amongst the mGlu receptors include the first and third intracellular loops and the TMDs. The intracellular carboxy (C)-terminus can vary in length, and alternative splicing of this region results in splice variants (a-e) of $mGlu_1$, and (a-b) of $mGlu_4$, $mGlu_5$, $mGlu_7$ and $mGlu_8$ (Pin et al., 1999).

Metabotropic Glu receptor ligand binding domains share a structural similarity with bacterial periplasmic binding proteins (Figure 1.4; O'Hara et al., 1993). Mutagenesis studies have shown that the extracellular N-terminus confers glutamate binding, agonist activation, and subtype specificity for selective agonists (O'Hara et al., 1993; Takahashi et al., 1993; Tones

et al., 1995). For example, the splice variant $mGlu_{4a}$ with a truncated N-terminus results in differential agonist potency compared to its full-length receptor (Han and Hampson, 1999). From the extensive work by Pin and colleagues an hypothesis has emerged to explain how the binding of the agonist in the extracellular domain accounts for the activation of the seventh TMD (Pin and Duvoisin, 1995; Bockaert et al., 1993; Gomeza et al., 1996a,b; Prezeau et al., 1996; Flor et al., 1996). The extracellular domain of mGlu receptors is generally accepted to be composed of two globular lobes with a hinge region where ligands are thought to bind (Figure 1.4B; Takahashi et al., 1993). The binding of an agonist in the large extracellular domain induces the closure of the two lobes. Originally it was thought that the agonist was transported to a second site in the seventh TMD where the agonist stabilises the active conformation of this region. However, functional studies utilising chimeric forms of mGlu receptors in the presence of Glu and Ca^{2+} do not support this hypothesis (Tones et al., 1995). Instead, a second, more probable hypothesis has been proposed. In this second hypothesis, the closed form of the extracellular binding domain, following agonist binding, activates the seventh TMD without relocating the agonist (Parmentier et al., 1998).

Homology and mutagenesis studies indicate that the amino acids Ser^{165} and Thr^{188} are conserved throughout the entire mGlu receptor family, including those mGlu receptors of the *Drosophilia* and *Caenorhabditis elegans*; these two residues are thought to form hydrogen bonds with the α-carboxylic and α-amino groups of Glu ligands respectively (O'Hara et al., 1993). The residues Arg^{57} and Ser^{167}, which are not uniformly conserved, may play a role in selective ligand recognition at group II mGlu receptors rather than participate in Glu binding (Pin et al., 1999). While the extracellular domain plays a role in the pharmacological profile of the receptor, but does not modify the G-protein coupling mechanism (Parmentier et al., 1998). A schematic of the mGlu receptor is shown in Figure 1.4.

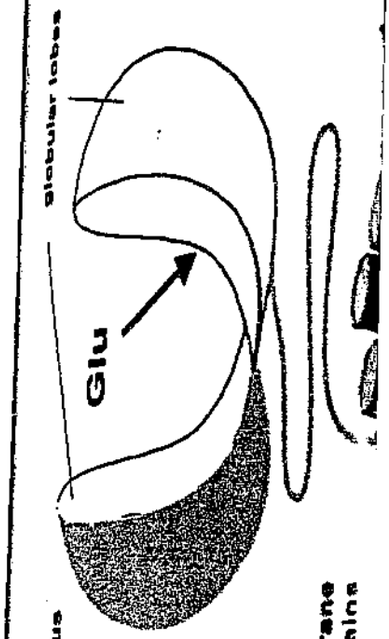
Figure 1.4 Structure of cloned mGlu receptors.

Three dimensional 'Ribbon' (A) and module (B) diagrams representing key aspects of the structure of mGlu receptors. Diagrams are constructed according to predicted folding patterns based on probabilities from what is known about amino acid residue assembly and hydrophilic and hydrophobic regions.

A. The seven TMD is represented in blue and the extracellular ligand binding domains are represented in yellow.

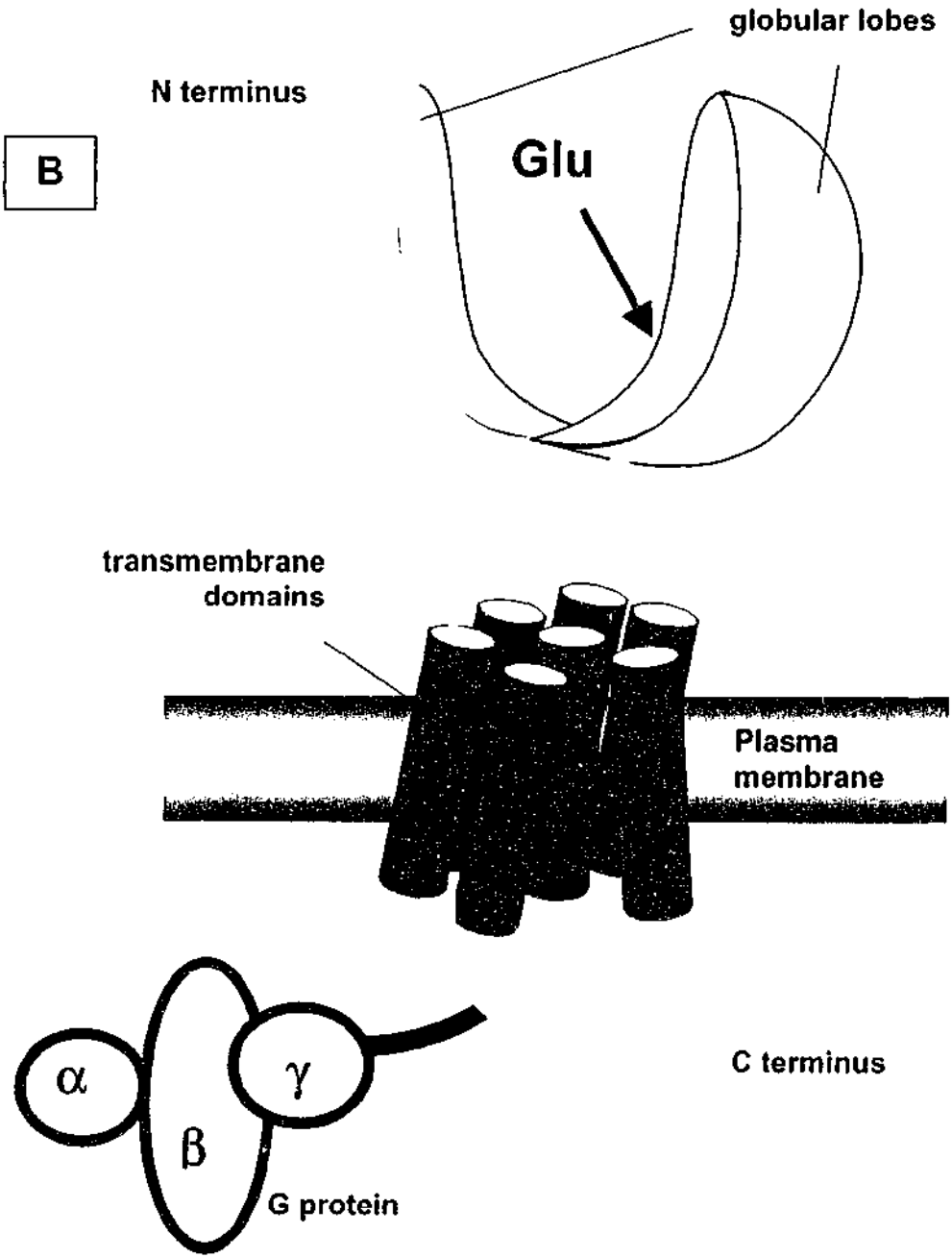
B. A similar construction of mGlu receptors shows where Glu binds to the receptor and where the G protein units positively couple.

Images courtesy of J.P. Pin, Montpellier, France.



B

A


globular lobes
N terminus
B
Glu
transmembrane
domains
Plasma
membrane
α
γ
β
G protein
C terminus

Studies of the molecular determinants involved in the coupling of family 1 GPCRs and G proteins indicate that the cavities in the second and third intracellular loops of the receptors come into contact with both the α- and βγ-subunits of the G protein. Chimeric studies have demonstrated that, like family 1 GPCRs, the second intracellular loop is critical for the transduction mechanism and G protein specificity of mGlu receptors (Gomeza et al., 1996b; Prezeau et al., 1996). For example, it is known that the G protein subunit isoforms $G\alpha_{15}$ and $G\alpha_{16}$ activate PLC of a number of GPCRs, including group I mGlu receptors. However, when these subunits are co-transfected with $mGlu_2$ or $mGlu_4$ (which normally activate AC) a PLC-mediated response is produced upon agonist activation of the receptors (Gomeza et al., 1996b). Considerably more research has been devoted to the interaction of the receptor with the C-terminus of the G_α-subunit than the N-terminus or the α4→β6 loop of the G protein. Consequently a number of residues in the C-terminus of the α-subunit have been identified as influencing G protein coupling to the receptor. One particular study identified that cysteine at the residue position Cys^{-4} of the C-terminus of the α-subunit was compatible with coupling of $G_{\alpha qi}$ and $G_{\alpha qo}$ to either $mGlu_2$ or $mGlu_4$, however replacing this residue with isoleucine favoured coupling of these subunits to $mGlu_4$ but not $mGlu_2$ (Blahos et al., 1998). It is evident from such research that (1) binding of a ligand in the extracellular N-terminus lobes of mGlu receptors does not influence G protein coupling or transduction pathways, (2) the G_α-subunit coupled to the mGlu receptors can influence the type of second messenger activated in the transduction pathway, and (3) residues in the C-terminus of the α-subunit influence G protein coupling to the receptor. Finally, while $mGlu_5$ has been shown to assemble into homodimers (Romano et al., 1996), the dimerisation of other mGlu receptors is at present unclear, with

some evidence demonstrating dimerisation involving group II mGlu receptors (Copani et al., 2000).

Recent focus on mGlu receptor function has come from the discovery of a number of functionally interacting intracellular proteins such as post-synaptic density (PSD) proteins (e.g. PSD-95), Homer, PICK and calmodulin. The structure of PSD-95 revealed a new protein motif, the PSD-95 disc-large zona occludens 1 (PDZ) domain, that plays an important role in the assembly of signal transduction complexes at intercellular junctions (Kennedy, 1998). In fact, tamalin (or GRP1-associated scaffold protein (GRASP)), is a PSD protein containing the PDZ domain which has been found to directly link $mGlu_1$ to guanine nucleotide exchange factors in yeast expression systems, and is co-localised with $mGlu_1$ in the telencephalon (Kitano et al., 2002).

Homer proteins (Homer 1a, b and c, Homer 2 and Homer 3) are members of the PDZ protein family and have been cloned from mouse, *Drosophila* and human. Homer specifically binds to mGlu receptors (Kato et al., 1998; Sun et al., 1998; Brakeman et al., 1997), and current understanding is that Homer protein dimers are formed that create physical links between $mGlu_{1a}$, $mGlu_{5a}$ or $mGlu_{5b}$ to IP_3 receptors, probably via Shank proteins, inducing Ca^{2+} release from intracellular stores (Fagni et al., 2000; Xiao et al., 1998; Tu et al., 1998; Ciruela et al., 2000). Shank is also thought to act with Homer to couple group I mGlu receptors to NMDA receptors (Lim et al., 1999; Sala et al., 2001).

Metabotropic glutamate receptors have also been associated with coupling to protein kinase C substrates, for example with $mGlu_7$ and "protein interacting c-kinase"-I (PICK1) (El Far et al., 2000; Boudin et al., 2000; Dev et al., 2000; Boudin and Craig, 2001; Dev et al., 2001). This interaction is specifically mediated by the distal C-terminal amino acids of the $mGlu_7$, and PICK1 is required for specific inhibition of P/Q-type Ca^{2+} channels in cultured cerebellar

granule cells, which may explain observations whereby PICK1 regulates the presynaptic function of $mGlu_{7a}$ to inhibit neurotransmission (Perroy et al , 2002).

Calmodulin (CaM) interacts with the C-terminal tail of $mGlu_{1a}$, $mGlu_5$ and $mGlu_7$ in a Ca^{2+}-dependent manner (O'Connor et al., 1999; Minakami et al., 1997). Binding of CaM is thought to disrupt G protein coupling by inducing release of the βγ subunit and possibly stimulating atypical signal transduction (O'Connor et al., 1999). In the case of $mGlu_5$ and $mGlu_7$, this interaction is prevented by PKC-induced receptor phosphorylation.

Although it is well known that the PKA activity is regulated by mGlu receptor function, recent studies have shown that PKA is capable of modulating mGlu receptor function via direct phosphorylation of serine sites in the C-terminus (Schaffhauser et al., 2000; Cai et al., 2001; Sorensen et al., 2002). The role of PKA and cAMP is dealt with in more detail in Chapter 3.

G protein-coupled inwardly rectifying K^+ channel (GIRK) currents are characterised by rapid activation and deactivation upon agonist application and removal, G protein dependence, strong inward rectification, Cs^+ and Ba^{2+} sensitivity, and K^+ selectivity. Previously, GIRK currents have been associated with dopamine D2 receptors and have only recently been shown to be associated with mGlu receptors, at first in *Xenpus Oocytes* (Saugstad et al., 1996; Sharon et al., 1997) and then in hippocampal neurones (Luscher et al., 1997). GIRK currents have been demonstrated for all three groups of mGlu receptors (Saugstad et al., 1996; Luscher et al., 1997; Dutar et al., 1999; Dutar et al., 2000; Sorensen et al., 2002). For example in unipolar brush cells group II mGlu receptors can functionally couple to activation of GIRK currents (Knoflach and Kemp, 1998). However, in Golgi cells of the cerebellum and interneurones of the accessory olfactory bulb, which also express group II mGlu receptors, agonists have not been shown to induce GIRK current activation despite inhibiting voltage-

gated Ca^{2+} channel currents (Knoflach and Kemp, 1998). A more comprehensive review of mGlu receptor interactions with ion channels has been published (Anwyl, 1999).

1.4 mGlu RECEPTOR EXPRESSION

Metabotropic glutamate receptors, like iGlu receptors, are widely distributed throughout the CNS. However, there appears to be less variable expression between the splice variants of mGlu receptor subtypes within most brain regions, when compared with the expression seen amongst iGlu receptor subtype splice variants (Wisden et al., 2000; Petralia et al., 2000; Shigemoto and Mizuno, 2000). Table 1.5 and Table 1.6 describe the level of mGlu receptor mRNA and protein expression, respectively, in selected brain regions.

1.4.1 *In vivo*

Distribution of $mGlu_1$ mRNA has been extensively mapped using *in situ* hybridisation histochemistry (Kerner et al., 1997; Masu et al., 1991; Shigemoto et al., 1992; Fotuhi et al., 1994). High $mGlu_1$ mRNA expression was shown in Purkinje cells of the cerebellum, the olfactory bulb, neocortex and various nuclei of the midbrain, thalamus and limbic cortex. High $mGlu_1$ protein expression was found in the molecular layer of the cerebellum, the glomerular layer of the main olfactory bulb and the islands of Calleja complex.

Developmental expression has been characterised for $mGlu_5$ (Romano et al., 1996). A decrease in $mGlu_5$ was found to occur either gradually or from postnatal day 18 in the brainstem, cerebellum, hypothalamus and the midbrain, while expression in the forebrain remained high. This decrease may be attributable to the loss of expression of the $mGlu_{5a}$ splice variant. Measurements of $mGlu_1$ and $mGlu_5$ immunolabelling reveal a decrease in expression with increasing distance from synaptic junctions and that the receptors tend to be found within

Table 1.5 Distribution of mGlu receptor mRNA in the adult rat CNS

Relative grain densities on neuronal cell bodies

	$mGlu_1$	$mGlu_5$	$mGlu_2$	$mGlu_3$	$mGlu_4$	$mGlu_7$	$mGlu_8$
Olfactory system: Main olfactory bulb	+~+++	+	+	-	-~+	+~+++	+++
Accessory olfactory bulb	+~+++	++	++	-	+~++	+~+++	++
Neocortex	+~+++	+~+++	+~+++	+~++	+	++	+
Limbic cortex:	+~+++	++~+++	++	++	+~++	++	+~++
Hippocampus	+++	+++	-	-	+~++	++	+
Dentate granule cells	++	++	++	++	+	++	+
Septal and basal forebrain regions	++	+~+++	+	-	+	++	+
Amygdala	+	++	++	+	-~+	++	+
Basal ganglia: Striatum	++	+++	+(s)	+	+	++	
Nucleus accumbens	+	+++	+	-	+	++	
Substantia nigra	+++	+	-	-	-	-~+	
Thalamus	++~+++	+	++	-	++	++	+
Reticular nucleus	-	+	-	+++	-	++	+
Hypothalamus	+~+++	+~++	-	+	-	++	-
Midbrain	++	+~++	-	++	-~+	++	-
Pons and Medulla oblongata	+	-~++	-	-	+	+~++	-
Cerebellum: Cerebellar cortex	++~+++	-	-	-	-	+~++	+
Golgi cells	+ ++	++	++++	++	++	+	-
Spinal cord: Dorsal horn	+	++	+(s)	+(s)	+	++	-
Ventral horn - Motor neurons	++	-	-	-	++~+++	+	-

A summary of the expression of the mGlu receptor subtype in each brain region is made, and particular nuclei of contrasting expression or those of interest have been included.
Relative grain densities: ++++ = very high; +++ = high; ++ = moderate; + = low; - = background level. (S) indicates that labelled cells are scattered in that region. ~ indicates varying labelling, e.g. +~+++ = low to high.
Abridged from Shigemoto and Mizuno, 2000.

Table 1.6 Distribution of mGlu receptor-like immunoreactivity in CNS

Density of immunoreactivity in neuropil

	$mGlu_1$	$mGlu_5$	$mGlu_2$	$mGlu_3$	$mGlu_4$	$mGlu_7$	$mGlu_8$
Olfactory system	++~+++	+~++	++	+++	+	++ (a)	+~++
Main olfactory bulb	++~+++	+	++	+/-	+	++ (a)	+~++
Accessory olfactory bulb	+~++	++	++	++	+	+~+++ (a)	+~++
Neocortex: Layer I-VI	+~++	++~+++	+++	+++	+~++	+~+++	++
Limbic cortex	+	++	++	+++	+~++	++~+++ (a)	+
Hippocampus	-/+	+++	++	++	++	++ (a)	++
Dentate gyrus	++	+~+++			+	+++	+
Septal and basal forebrain regions	+	+	+	+	+	+~++	-
Amygdala	+	+	++~+++	+~+++	+~++	++~+++ (a)	++
Basal ganglia: Striatum	+~++	++++	+++	+++	+~++	+ (a)	++
Nucleus accumbens	+	++++	+++	+++	++	+ (a)	-
Substantia nigra	+~+++	-	+	+	+	+~++	+
Thalamus	++	+	+~+++	+	+~++	+ (a)	-
Hypothalamus	+	-~+	-	+	+~++	+ (a)	-
Midbrain	++	+	-~++	+	+~++	+ (a)	-
Pons and Medulla oblongata	+~++	+	-~+++	+	+~++	+ (a)	-
Cerebellum: Molecular layer	++++	-	-	+	+++	-	++
Purkinje cell layer	+++	-	-	-	-	+ (b)	+
Granule cell layer	+	+	+++	+/-	+	-	++
Cerebellar nuclei	++	++	+	+/-	+	++ (b)	-
Spinal cord: Dorsal horn	++	++	++	+	+~++	+~+++	-
Ventral horn	+	+	-	-	+~++	-	-

A summary of the expression of the mGlu receptor subtype in each brain region is made, and particular nuclei of contrasting expression or those of interest have been included.
Relative grain densities: ++++ = very high; +++ = high; ++ = moderate; + = low; - = background level. ~ indicates varying labelling, e.g. +~+++ = low to high.
For mGlu7 expression, (a) or (b) represents the expression for that particular isoform only. When the alternative isoform is not noted there was no receptor expression. When neither isoforms are given then the expression level applies to both isoforms.
Abridged from Shigemoto and Mizuno, 2000.

the PSD (Lujan et al., 1996). Of the group I mGlu receptors, only $mGlu_5$ appears to be expressed on astrocytes *in vivo* (van den Pol et al., 1994; van den Pol et al., 1995).

Group II mGlu receptors are generally distributed throughout the forebrain and cerebellar cortex with both pre- and post-synaptic localisation (Neki et al., 1996a). While $mGlu_2$ immunoreactivity is extensive throughout the olfactory bulb, cerebral cortex and caudate-putamen, this receptor subtype appears to be only expressed in the Golgi cells of the cerebellum (Genazzani et al., 1993; Ohishi et al., 1993b). Interestingly, $mGlu_2$ and $mGlu_5$ are expressed in two separate populations of Golgi cells, approximately 90% of which were found to be $mGlu_2$ immunoreactive and 10% $mGlu_5$ immunoreactive (Neki et al., 1996b). The $mGlu_3$ subtype is similarly expressed on neurones throughout the forebrain and cerebellum, but is also uniquely located on glia (Ohishi et al., 1993a; Ohishi et al., 1994; Jeffery et al., 1996; Mineff and Valtschanoff, 1999). While no $mGlu_3$ mRNA is evident in cerebellar granule cells (Ohishi et al., 1993a), positive immunoreactivity is evident for the receptor in this layer (Ohishi et al., 1994) indicating a possible mechanism for the transport of the protein.

The group III mGlu receptor, $mGlu_6$, is only expressed in the retina (Nakajima et al., 1993; Shigemoto and Mizuno, 2000). Localisation of $mGlu_4$ has been reported in the rat cerebral cortex and hippocampus (Phillips et al., 1997), and further studies show this subtype to be localised on the pre- and postsynaptic terminals (Kinoshita et al., 1996). The splice variants $mGlu_{7a}$ and $mGlu_{7b}$ are similarly distributed in rat and mouse where $mGlu_{7a}$ appears more widespread and includes sensory pathways. Both $mGlu_7$ splice variants are found on non-glutamatergic axon terminals including those that are presumed to be inhibitory (Kinoshita et al., 1998). The expression of $mGlu_8$ appears to be confined to the olfactory bulb, and expressed to a lesser extent in the piriform cortex and dentate gyrus (Saugstad et al., 1997; Corti et al., 1998; Shigemoto and Mizuno, 2000). In the main olfactory bulb, $mGlu_8$ expression

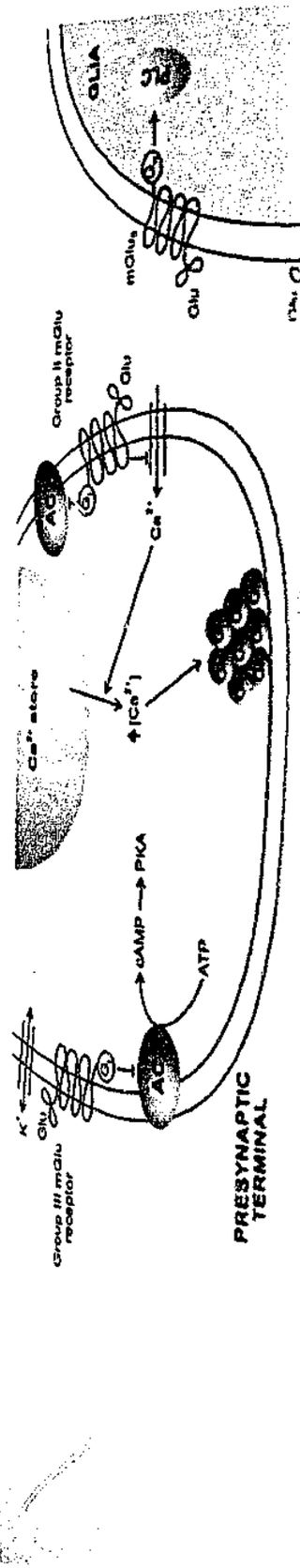
is greater in the mitral cell layer compared to the granule cell layer, however the inverse is true in the accessory olfactory bulb. Pre- and postsynaptic expression of $mGlu_8$ has been mapped in the retina (Koulen and Brandstatter, 2002) and is thought to contribute to the negative feedback mechanism involved in the fine adjustment of the light-regulated release of glutamate from photoreceptors. Figure 1.5 demonstrates the cellular localisation of mGlu receptors and respective second messenger coupling mechanisms. At present, no evidence exists for marked differences in mGlu receptor protein or mRNA expression between rat and mouse brain.

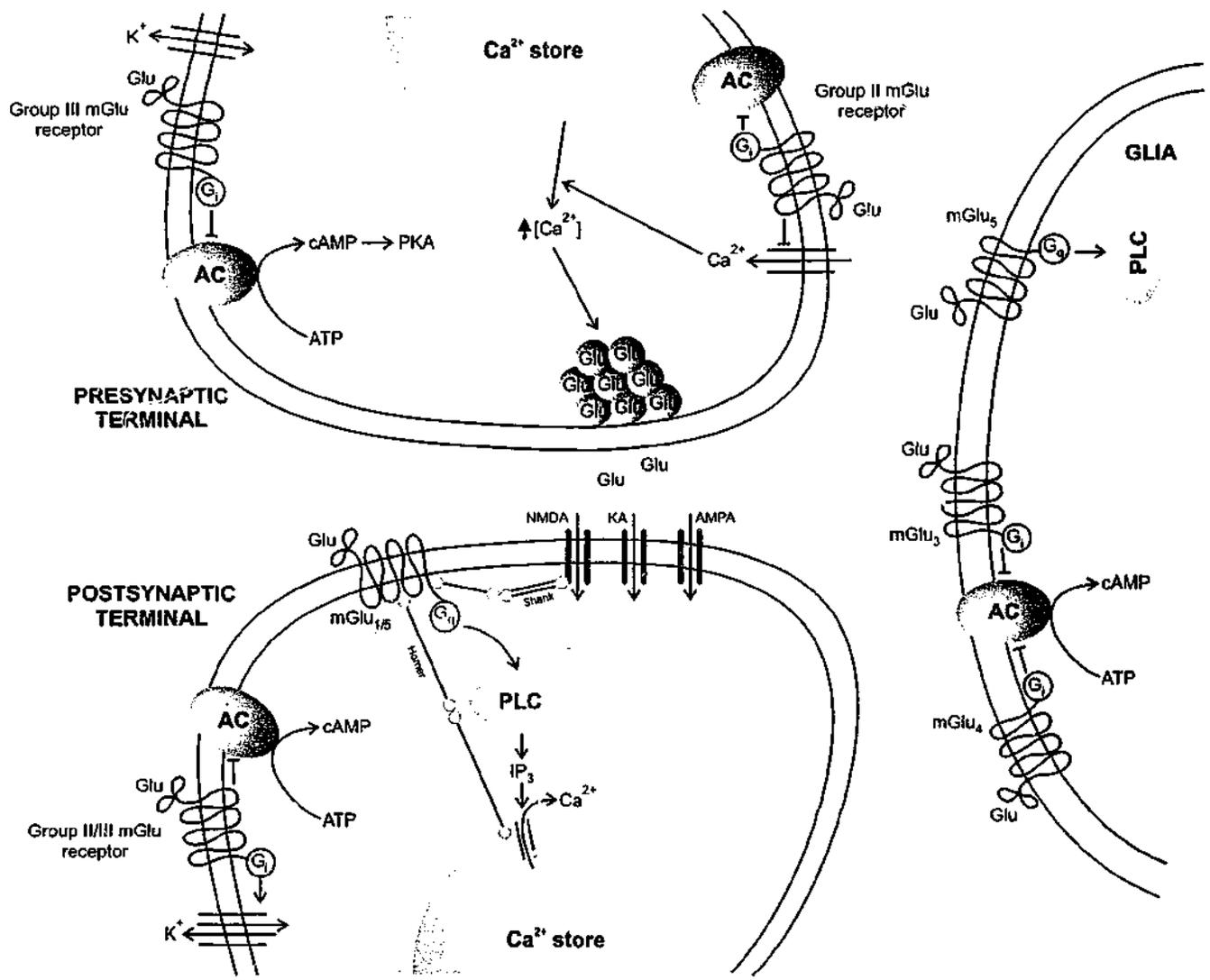
In general, human mGlu receptor expression correlates well with that in rat brain, particularly in the cerebellum where extensive expression studies have been undertaken (Makoff et al., 1996a,b,c; Blumcke et al., 1996; Makoff et al., 1997; Berthele et al., 1999). In such studies mRNA coding for mGlu subtypes 1, 3, 4 and 7 were expressed in Purkinje cells and mGlu subtypes 1 and 4 were additionally expressed in granule cells; subtypes 1, 2, 3, 4 and 7 were detected in Golgi cells. Bergmann glial cells express $mGlu_5$ and $mGlu_3$ mRNA. Expression of $mGlu_8$ mRNA expression was low in the molecular layer and $mGlu_6$ mRNA was not detectable.

In addition to the cerebellum, *in situ* hybridisation studies with human brain sections have showed $mGlu_3$, $mGlu_4$ and $mGlu_7$ mRNA expression on neurones of the cerebral cortex, caudate-putamen and thalamus (Makoff et al., 1996a,b,c; Richardson-Burns et al., 2000). In the hippocampus, $mGlu_{1a}$ and $mGlu_7$ is moderately expressed in the dentate gyrus and CA1, with more diffuse labelling in the CA2 and CA3 regions; and diffuse labelling exists for $mGlu_5$ in all hippocampal regions (Blumcke et al., 1996; Malherbe et al., 1999; Blumcke et al., 2000). The pattern of human $mGlu_{8a/b}$ mRNA expression was not found to be different between adult and foetal human brain, with predominant expression in the olfactory bulb (Malherbe et al., 1999).

Figure 1.5 Schematic representation of the localisation and coupling of mGlu receptor subtypes at a glutamatergic synapse.

Group I mGlu receptors are predominantly localised on the postsynaptic terminal where $mGlu_5$ has been shown to couple to NMDA receptors via intracellular proteins Shank and Homer, or to intracellular calcium stores via Homer. Some evidence exists for presynaptic localisation of $mGlu_{1/5}$. Group II and III mGlu receptors are located on the pre- and postsynaptic terminal in a perisynaptic position and appear to be recruited in times of high synaptic concentrations of Glu. The receptor subtypes $mGlu_3$, $mGlu_5$ and more recently, $mGlu_4$, have been found on astrocytes. (Cartmell and Schoepp, 2000; Tamaru et al., 2001)




K+
Glu
Group III mGlu receptor
Gi
AC
cAMP → PKA
ATP
PRESYNAPTIC TERMINAL
Ca2+ store
↑[Ca2+]
Ca2+
AC
Group II mGlu receptor
Gi
Glu
Glu
Glu
Glu
Glu
Glu
Glu
Glu
Glu
Glu
NMDA
KA
AMPA
Glu
POSTSYNAPTIC TERMINAL
mGlu1/5
Gq
Shank
Homer
PLC
IP3
Ca2+
AC
cAMP
ATP
Glu
Group II/III mGlu receptor
Gi
K+
Ca2+ store
GLIA
mGlu5
Gq
PLC
Glu
Glu
mGlu3
Gi
AC
cAMP
ATP
Gi
mGlu4
Glu

The major difference from the rat brain is the presence in the human brain of $mGlu_4$ mRNA in the caudate nucleus and putamen (Makoff et al., 1996b). A predominantly glial cell expression exists for an $mGlu_8$ isoform in human brain ($mGlu_{8c}$) (Malherbe et al., 1999).

Immunocytochemical analysis has demonstrated that neuronal expression of group I mGlu receptors is high throughout the spinal cord with highest expression of $mGlu_{1a}$ and $mGlu_5$ in the ventral horn and dorsal horn, respectively (Aronica et al., 2001). Group II mGlu receptor immunoreactivity was mainly concentrated in the inner part of the lamina II. In spinal cord, glia showed weak and moderate immunoreactivity of $mGlu_5$ and $mGlu_{2/3}$, respectively (Aronica et al., 2001).

1.4.2 *In vitro*

In cortical neuronal cultures mRNA and protein expression of the mGlu subtypes 1, 3, 4, 5, 6, 7, and 8 are quite similar (Janssens and Lesage, 2001), but no $mGlu_2$ mRNA expression was detected, in contrast to the study conducted by Heck et al. (1997). Such subtle differences may reflect the age of the cultures used, or the growth medium employed. In cultures of the hippocampus and cerebellum all mGlu receptor subtype mRNA is expressed, however $mGlu_{1/5}$ protein levels appear higher than $mGlu_{2/3}$, while $mGlu_4$ and $mGlu_8$ protein were absent (Aronica et al., 1993; Janssens and Lesage, 2001). In cortical astrocytes grown with or without serum, $mGlu_3$ and $mGlu_5$ mRNA and protein has consistently been found to be present (Miller et al., 1995; Ciccarelli et al., 1997; Nakahara et al., 1997; Janssens and Lesage, 2001). While a majority of research has found no evidence of any other mGlu receptor subtype mRNA or protein on cortical astrocytes, Nicoletti and colleagues recently found positive immunoreactivity and mRNA expression of $mGlu_4$ (Besong et al., 2002).

1.5. mGlu RECEPTOR PHARMACOLOGY

Apart from Glu, initial agonists employed to study mGlu receptors in a number of model systems included quisqualate and ibotenate (Figure 1.6; Palmer et al., 1989; Baird et al., 1991) However these compounds are also agonists at iGlu receptors, hence, the discovery of the conformationally restricted Glu analogue *t*-ACPD, which stimulated PI hydrolysis, represented the first evidence for a selective mGlu receptor agonist (Schoepp and Hillmán, 1990; Manzoni et al., 1990; Schoepp et al., 1991a) (chemical names of all drugs appear in Abbreviations). Furthermore, the mGlu receptor agonist activity of *t*-ACPD was found to reside predominantly in the 1*S*,3*R*-isomer (1*S*,3*R*-ACPD) (Irving et al., 1990; Schoepp et al., 1991a; Schoepp et al., 1992a; Cartmell et al., 1993). Investigations into mGlu receptor function employing 1*S*,3*R*-ACPD revealed that this isomer was non-selective (Cartmell et al., 1993; Schoepp et al., 1992a), possessing similar agonist potency across all mGlu receptors except $mGlu_7$ (Flor et al., 1997). A number of selective agonists and antagonists now exist for group I and II mGlu receptors, prompting diverse studies into the role of these receptors in physiological and pathological conditions (discussed in detail below).

Activation of presynaptic protein kinase C (PKC) with phorbol esters enhances glutamate release. However, it has been shown that the non-selective mGlu receptor agonist *t*-ACPD mediates an enhancement of glutamate exocytosis via the DAG-PKC pathway that is dependent on arachidonic acid, possibly through the synergistic activation of PKC (Birrell et al., 1993; Vazquez et al., 1994; Vazquez et al., 1995; Blanc et al., 1995). Modulation of glutamate release in this way has been shown to be important in long-term potentiation (LTP) (Herrero et al., 1992) which is implicated in learning and memory.

Group I and II mGlu receptors have been shown to modulate cardiovascular effects (Li et al., 1999; Jones et al., 1999), induce hepatic glucose production, and stimulate adrenal

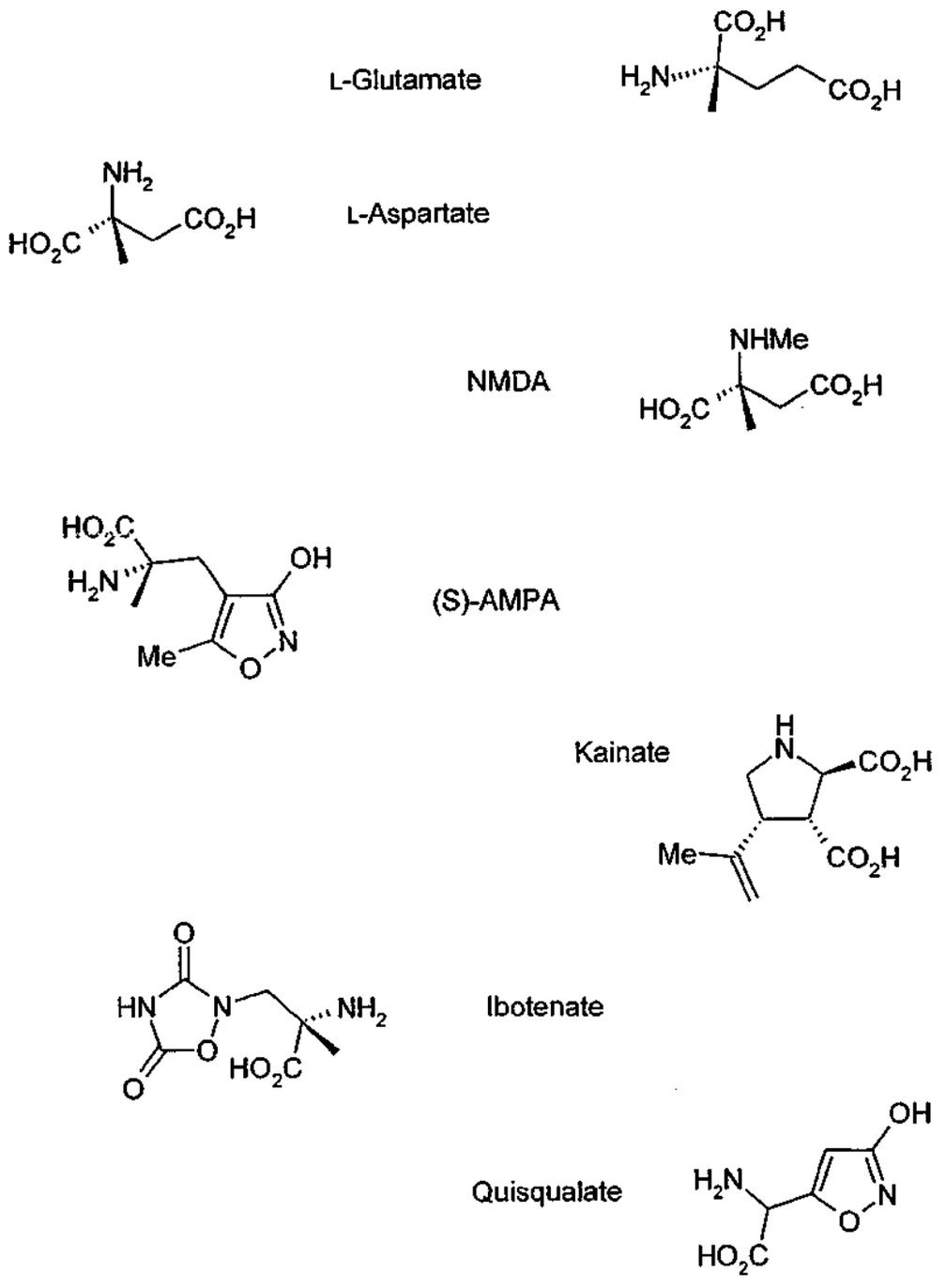


Figure 1.6 Classical Glu receptor ligands. Me: methyl group.

and pancreatic hormone secretion (Lang and Ajmal, 1995). Activation of mGlu receptors has also been linked to genomic responses. For example, *t*-ACPD has been shown to cause a transient increase in immediate early genes (IEG) c-fos, c-jun, and zif-268 in primary neuronal cultures (Condorelli et al., 1994).

1.5.1 Group I mGlu receptors

1.5.1.1 *Agonists*

Early group I mGlu receptor studies employed 1*S*,3*R*-ACPD. However due to its non-selective activity, which is shared by its more recent constrained analogue ABH x D-I (Kozikowski et al., 1998), any interpretation from these early functional and pharmacological studies must be undertaken with caution. DHPG was the first agonist shown to be group I mGlu receptor selective, displaying no agonist or antagonist activity at native (Schoepp et al., 1994) or recombinant rat and/or human group II and III mGlu receptors (Gereau and Conn, 1995; Wu et al., 1998). The agonist activity of DHPG resides in the *S*-isomer (Baker et al., 1995) and is only slightly more selective for $mGlu_5$ over $mGlu_1$ (Ito et al., 1992; Brabet et al., 1995). The agonist, CHPG, which is an analogue of DHPG has been found to be more selective for $mGlu_5$, but lacks the potency of DHPG. Group I mGlu receptor agonists generally display the following rank order of potency: quisqualate > ABH x D-I > DHPG = Glu > DHPMP > 1*S*,3*R*-ACPD > L-CCG-I > 3-HPG > CHPG > *t*-ADA.

Group I mGlu receptors are known to be involved in the modulation of the release of Glu, GABA, dopamine and substance P (see Table 1.7). However, an increase in the release of 5HT and acetylcholine (ACh), or a decrease in the release of purines and cholecystokinin has been reported with the non-selective agonist 1*S*,3*R*-ACPD (reviewed by Cartmell and Schoepp, 2000). In general, DHPG has been used to demonstrate that activation of group I

Table 1.7 Effects of selective mGlu receptor agonists on neurotransmitter release. Most of these effects were under stimulated or depolarising conditions (Abridged from Cartmell and Schoepp, 2000).

Pharmacology	Release	Preparation	Reference
group I agonists	↑ Glu	rat cortical & hippocampal synaptosomes	(Herrero et al., 1998) (Rodriguez-Moreno et al., 1998)
	↑ GABA	rat NTS, striatal & hippocampal slices	(Jones et al., 1998) (Wang et al., 1996) (Janaky et al., 1994)
	↑ Dopamine	microdialysis: striatum, NAc	(Taber et al., 1995) (Bruton et al., 1999)
	↓ Substance P	rat trigeminal nucleus slices	(Cuesta et al., 1999)
group II agonists	↓ Glu	rat cortical & hippocampal synaptosomes microdialysis: striatum	(Allen et al., 1999) (Battaglia et al., 1997) (Di Iorio et al., 1996)
	↓ GABA	cortical neurons NTS or striatal slices	(Schaffhauser et al., 1998) (Jones et al., 1998) (Hanania and Johnson, 1999)
	↓ Dopamine	rat NAc	(Hu et al., 1999)
	↑ Dopamine	rat striatum	(Bruton et al., 1999)
	↑ 5HT	rat PAG	(Maione et al., 1998)
	↓ ACh	rat striatal slices	(Hanania and Johnson, 1999)
	↓ Purines	rat hippocampal slices	(Di Iorio et al., 1996)
	↑ Taurines	mouse hippocampal slices	(Saransaari and Oja, 1999)
group III agonists	↓ Glu	rat cortical synaptosomes	(Herrero et al., 1996) (East et al., 1995)
	↓ GABA	cultured rat cortical & striatal neurons	(Schaffhauser et al., 1998) (Lafon-Cazal et al., 1999a)
	↓ Dopamine	microdialysis: rat NAc	(Hu et al., 1999)
	↑ 5HT	rat PAG	(Maione et al., 1998)
	↓ Substance P	rat trigeminal nucleus slices	(Cuesta et al., 1999)

Abbreviations: Ach, acetylcholine; 5HT, serotonin; NAc, nucleus accumbens; NTS, nucleus tractus solitarii; PAG, periaqueductal gray.

mGlu receptors increases Glu, GABA and dopamine release in cortical, striatal and hippocampal regions, while inhibiting substance P release in the trigeminal nucleus.

In addition to modulating neurotransmitter release, accumulating evidence has identified group I mGlu receptors as the predominant subtype that mediates mGlu receptor-induced EPSPs in the Purkinje cells of the cerebellum, corticothalamic projections and in the CA1 of the hippocampus (McGuinness et al., 1991; Crepel et al., 1994; Jouvenceau et al., 1995; Breakwell et al., 1996; Manahan-Vaughan and Reymann, 1996; Eaton and Salt, 1996; Batchelor et al., 1997; Anwyl, 1999). These observations give support to the role of group I mGlu receptors in the induction of LTP (Palmer et al., 1997; Manahan-Vaughan and Reymann, 1997). The involvement of mGlu receptors in LTP is complex (see review: Bortolotto et al., 1999). For example, in the above studies, DHPG-induced depression of the postsynaptic potential was described which led to the hypothesis that this latter response involved the postsynaptic release of arachidonic acid which could act in a negative feed-back manner at the presynaptic terminal. Subtype knock-out studies revealed that, despite their expression at CA1 synapses, both group I mGlu receptor subtypes are not regarded as essential for AMPA receptor-mediated LTP but are involved to differing degrees in NMDA receptor-mediated LTP (Jia et al., 1998; Lu et al., 1997). At one time a molecular switch was proposed for the apparent duality of the LTP responses (Bortolotto et al., 1994), later evidence for this switch implicated PKC and CaMKII activation following group I mGlu receptor stimulation by DHPG (Bortolotto and Collingridge, 1998; Bortolotto and Collingridge, 2000). More recently, intracellular proteins Shank and Homer have been hypothesised to be responsible for the group I mGlu receptor involvement with AMPA and NMDA receptor-mediated LTP (Daw et al., 2000; Cho et al., 2002).

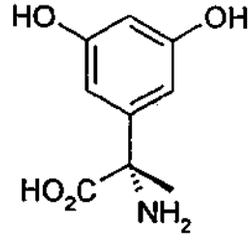
Finally, in cultured cortical astrocytes Bezzi et al. (1998) showed that DHPG and 1*S*,3*R*-ACPD increased Glu release, which was potentiated by AMPA through the release of prostaglandins.

1.5.1.2 *Antagonists*

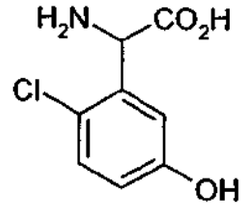
Group I mGlu receptor antagonists have been characterised as either competitive or non-competitive. Some of the early competitive antagonists were found to either possess agonist-dependent actions (e.g. L-AP3; Saugstad et al., 1995) or have partial group II mGlu receptor agonist activity themselves (e.g. the phenylglycine MCPG; Hayashi et al., 1994; Watkins and Collingridge, 1994). AIDA is a conformationally restricted analogue of MCPG which possesses greater selectivity for group I mGlu receptors than its parent. AIDA and a number of other similar analogues were found to fit a three-dimensional model of $mGlu_1$, and from such modelling studies it has been proposed that competitive group I mGlu receptor antagonists stabilise the open form of the receptor thus preventing the conformational movement required for closing of the extracellular lobes during agonist binding (Constantino and Pellicciari, 1996). Three more recently developed $mGlu_{1/5}$ antagonists which modulate the receptor rather than compete with Glu for its binding site are MPEP ($mGlu_5$ specific; Pagano et al., 2000), CPCCOEt and BAY36-7620 (Litschig et al., 1999; Hermans et al., 1998). The latter non-competitive antagonist has been employed to elucidate the constitutive activity of mGlu receptors and the individual roles of $mGlu_1$ and $mGlu_5$, respectively (Carroll et al., 2001). Key $mGlu_{1/5}$ agonists and antagonists are presented in Figure 1.7.

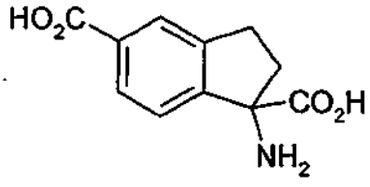
Agonists:

S-DHPG



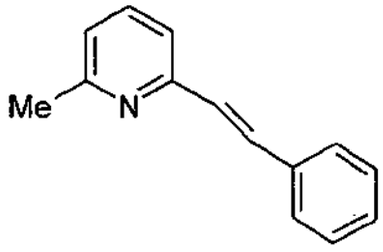
CHPG





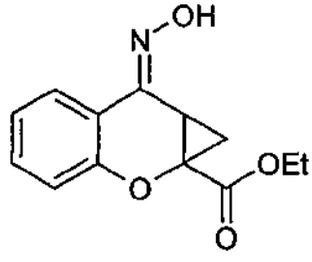
Antagonists:

AIDA



MPEP

BAY36-7620



(-)-CPCCOEt

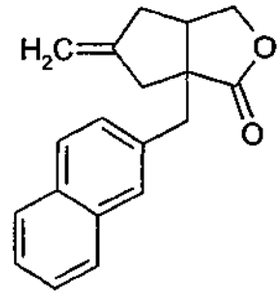


Figure 1.7 Chemical structures of group I mGlu receptor agonists and antagonists. Et: Ethyl group. Me: Methyl group.

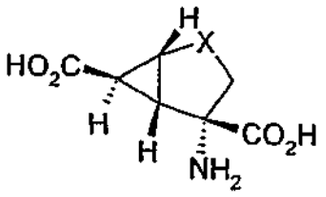
1.5.2 Group II mGlu receptors

1.5.2.1 *Agonists*

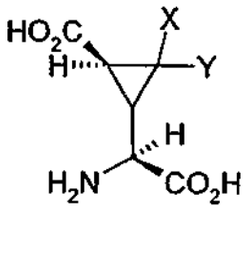
A systematic evaluation of eight enantiomers of 3,4-methyleneglutamic acid led to the discovery of L-CCG-I, a highly potent and selective group II mGlu receptor agonist (Hayashi et al., 1992; Lombardi et al., 1993; Prezeau et al., 1994). Until more recently, L-CCG-I derivatives (e.g. DCG-IV and *cis*-MCG-I) represented the most selective family of compounds for group II mGlu receptors. However the use of pharmacophore models has seen the development of conformationally constrained bicyclic and heterobicyclic ring compounds (e.g. LY354740 and LY379268, respectively) with greater selectivity and potency (Schoepp et al., 1997; Monn et al., 1997; Monn et al., 1999). At present, the following rank order of potency generally exists for group II selective agonists (established from recombinant studies): LY379268 = LY389795 > LY354740 = L-F2CCG-I > DCG-IV = L-CCG-I = *t*-MCG-I = 2*R*,4*R*-APDC = *S*-4MeGlu > *cis*-MCG-I > ABH x D-I > Glu = 2*S*,4*S*-4-MG = 1*S*,3*R*-ACPD = 1*S*,3*S*-ACPD > L-CBG-I. The group II agonist NAAG (or Spaglumic acid), has a potency similar to 1*S*,3*R*-ACPD, but is the most selective (5-fold) agonist for $mGlu_3$ over $mGlu_2$ (Wroblewska et al., 1997). Group II mGlu receptor agonists are presented in Figure 1.8.

Selective group II mGlu receptor agonists have been shown to modulate release of not only Glu, GABA and dopamine, but also 5HT, ACh, purines and taurines (see Table 1.7). In general group II mGlu receptors mediate the inhibition of Glu and GABA release from nerve terminals in the cortex and striatum. In normal physiological conditions activation of presynaptic inhibitory $mGlu_{2/3}$ is understood to occur when an increase in Glu concentration is present within the synaptic cleft. This mechanism contributes to a negative feedback pathway for controlling glutamatergic synaptic neurotransmission (Scanziani et al., 1997).

Heterobicyclics:

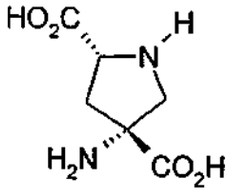


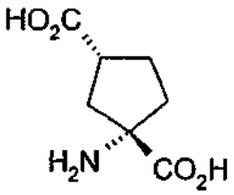
X	Name
CH_2	LY354740
O	LY379268
S	LY389795



X	Y	Name
H	H	L-CCG-I
H	CO_2H	DCG-IV
H	CH_2OCH_3	cis-MCPG-I
CH_2OCH_3	H	trans-MCPG

1*S*,3*S*-ACPD





2*R*,4*R*-APDC

NAAG

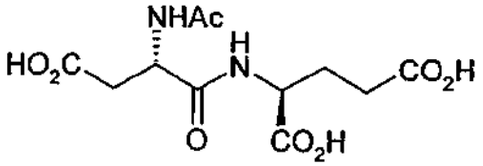


Figure 1.8 Chemical structures of group II mGlu receptor agonists.
Ac: Acetyl group.

Group II mGlu receptor agonists have been shown to inhibit EPSPs in a number of preparations (Macek et al., 1996; Flavin et al., 2000; Kilbride et al., 1998; Anwyl, 1999; Lea et al., 2001). This activity was shown to be via inhibition of Glu release from presynaptic terminals and most likely as a result of inhibiting VOCC. Group II mGlu receptors are also understood to play a role in the induction of long-term depression (LTD) of postsynaptic potentials. For example, the activation of group II mGlu receptors was required in addition to group I mGlu- and NMDA-receptor mediated LTD in the perirhinal cortex (Cho et al., 2000). By comparison, group II mGlu receptor agonists are able to independently induce LTD in the dentate gyrus (Huang et al., 1999; Lea et al., 2001) and the visual cortex (Renger et al., 2002). This inhibitory activity may reside with the $mGlu_2$ subtype since LTD in the mossy fibres of the hippocampus was impaired in those mice lacking $mGlu_2$ (Yokoi et al., 1996). Together with molecular biology studies and immunogold labelling demonstrating the close proximity of group I mGlu receptors to group II mGlu receptors on the postsynaptic membrane in these regions (Lujan et al., 1997), a theory has emerged which links the cAMP response induced by group II mGlu receptors to the phosphorylation of $mGlu_5$ via PKA (Cho et al., 2002). Through this receptor interaction group II mGlu receptors have been hypothesised to induce LTD and consequently, play an important role in synaptic plasticity.

Other experiments investigating neurotransmission have suggested that the $mGlu_{2/3}$-mediated presynaptic inhibitory mechanism may be shared with adenosine receptors (Di Iorio et al., 1996), and that group II mGlu receptor agonists may augment adenosine receptor-mediated responses (Ogata et al., 1994; Cartmell et al., 1997).

1.5.2.2 *Antagonists*

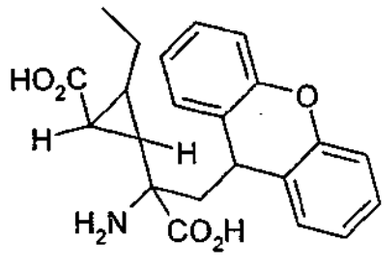
The first compound to be identified as an antagonist at group II mGlu receptors was MCPG. This compound, and its family of ω-carboxyl group substituted analogues, were found to have a weak antagonist activity at $mGlu_1$ and $mGlu_4$ (Thomsen et al., 1996; Kowal et al., 1998). A group of L-CCG-I analogues represented a second line of antagonists with greater group II mGlu receptor selectivity. One of these analogues, MCCG, was found to possess antagonist activity at both human and rat $mGlu_2$, and is without activity at human and rat $mGlu_1$, $mGlu_4$ and $mGlu_6$ (Laurie et al., 1997; Knopfel et al., 1995). Partial agonist activity in rat cortical slices has been noted with MCCG (Kemp et al., 1996) although the receptor subtype responsible for this activity has not been identified. FGlu, is a glutamate analogue with antagonist activity and selectivity for $mGlu_2$ (Jane et al., 1996). Non-competitive antagonists for group II mGlu receptors have not yet been reported. More recently, pharmacological characterisation using cloned human mGlu receptor subtypes has shown that LY341495 is the most potent group II antagonist developed (Ornstein et al., 1998; Kingston et al., 1998). Employing agonists and antagonists in human $mGlu_2$ expressing cells revealed the relative order of displacement for specific binding of [^{3}H]LY341495 was LY341495 > LY354740 > DCG-IV > L-CCG-I > 2*R*,4*R*-APDC = Glu > 1*S*,3*R*-ACPD > MCPG (Johnson et al., 1999). Group II mGlu receptor antagonists are presented in Figure 1.9.

1.5.3 Group III mGlu receptors

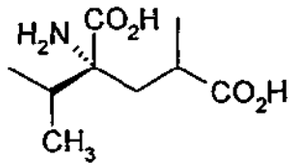
1.5.3.1 *Agonists*

The pharmacology of group III mGlu receptors is not as advanced as that of group I or group II mGlu receptors, which is partially due to the diversity of group III mGlu receptors. L-AP4 represents the most selective agonist of group III mGlu receptors, in

LY341495



EGlu



LY307452

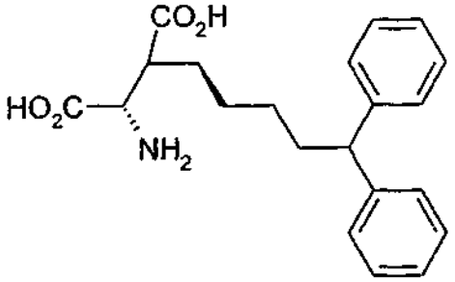


Figure 1.9 Chemical structures of group II mGlu receptor antagonists.

particular $mGlu_4$ (Tanabe et al., 1993) and $mGlu_6$ (Laurie et al., 1997), while the endogenous group III mGlu receptor agonist, L-SOP, exhibits partial antagonist activity at group II mGlu receptors (Pin et al., 1999). For a long time the agonist (*RS*)-PPG was thought to be the most potent and selective $mGlu_8$ agonist (Flor et al., 1998), however recent work by Thomas et al. (2001) shows that in transfected cell lines and rat spinal cord at least, the newly developed *S*-3,4-DCPG is more potent and selective. While more information is being accumulated regarding the activity of *S*-3,4-DCPG, *R,S*-PPG is regularly used as the standard $mGlu_8$ agonist. A general, rank order of potency can be assembled: *S*-3,4-DCPG ≥ L-AP4 ≥ *R,S*-PPG ≥ L-SOP > L-CCG-I > L-Glu > 1*S*,3*R*-ACPD.

The ability of group III mGlu receptors to modulate neurotransmission, and inhibit EPSPs, resembles that of the group II mGlu receptors, and probably derives from their presynaptic localisation and signal transduction mechanisms which are also shared with group II mGlu receptors (see Table 1.7; Macek et al., 1996; Anwyl, 1999). Presynaptic expression of $mGlu_8$ in the retina is thought to contribute to the negative feedback mechanism involved in the fine adjustment of the light-regulated release of glutamate from photoreceptors (Koulen et al., 1999). Similarly, $mGlu_6$ is strictly expressed at the postsynaptic site of ON-bipolar cells in both rod and cone systems, and $mGlu_6$ gene targeting experiments, which disrupt ON responses without changing OFF responses, severely impaired the visual responses of those animals (Nakanishi et al., 1998).

1.5.3.2 *Antagonists*

Non-competitive antagonists for group III mGlu receptors have not yet been reported. Present data regarding group III mGlu receptor competitive antagonists are conflicting, or non-selective activity is reported for group II mGlu receptors. Most of the current antagonists

were developed from modifications of mGlu receptor agonists. For example, it was noted that adding a carboxy group to L-CCG-I (to become DCG-IV) changes its activity from not only a group II mGlu receptor agonist but also to a group III mGlu receptor antagonist (Brabet et al., 1998). Furthermore, characterisation of group III mGlu receptor antagonists has tended to centre on the ability of the compound to inhibit L-AP4 depression of synaptic transmission in the neonatal rat spinal cord (Pook et al., 1993; Kemp et al., 1994; Cao et al., 1997a,b; Thomas et al., 2001). Two compounds that moderately inhibit this activity are the α-methyl analogues of the agonists L-SOP and L-AP4, which are termed MSOP and MAP4, respectively (Jane et al., 1994; Johansen and Robinson, 1995). A recently developed $mGlu_8$ specific antagonist UBP1111 is a methyl derivative of the 3,4-DCPG compounds, but has yet to be widely tested (Thomas et al., 2001). Key group III mGlu receptor agonists and antagonists are presented in Figure 1.10.

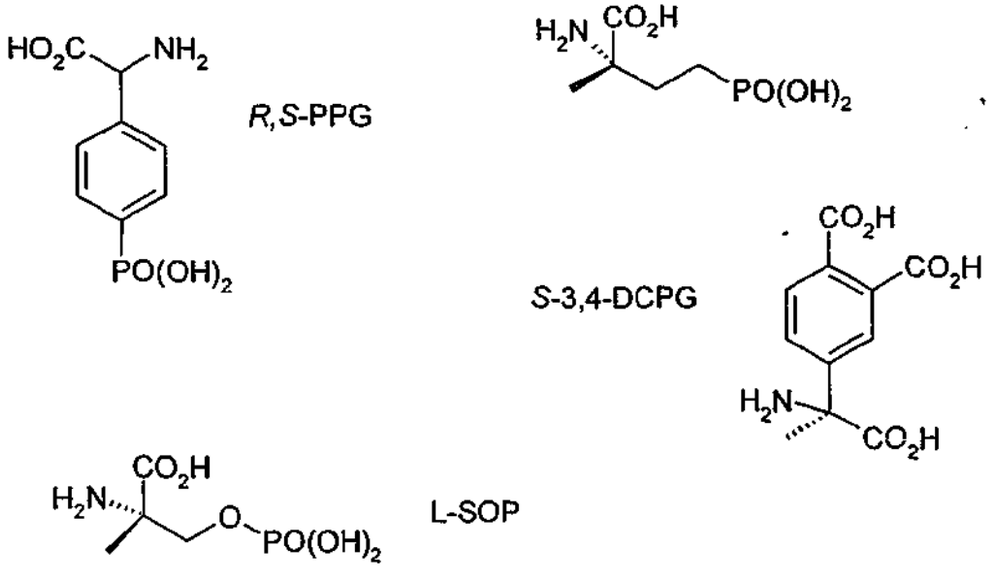
1.6. GLUTAMATE IN INJURY AND EPILEPSY

1.6.1 Glutamate and Excitotoxicity

1.6.1.1 *Historical Perspective*

Although Glu plays important roles in normal physiology, excessive stimulation of Glu receptors has been implicated in neuronal injury and death in numerous pathological conditions such as stroke, epilepsy or neurotrauma, and neurodegenerative diseases including Huntington's chorea, Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994; Leist and Nicotera, 1998). Evidence to suggest the involvement of Glu in various acute and chronic neurological dysfunctions has come from both *in vivo* and *in vitro* cytopathological evidence and from direct evidence, whereby high concentrations of Glu have been recovered from damaged brain areas

Agonists: L-AP4



Antagonists: MAP4

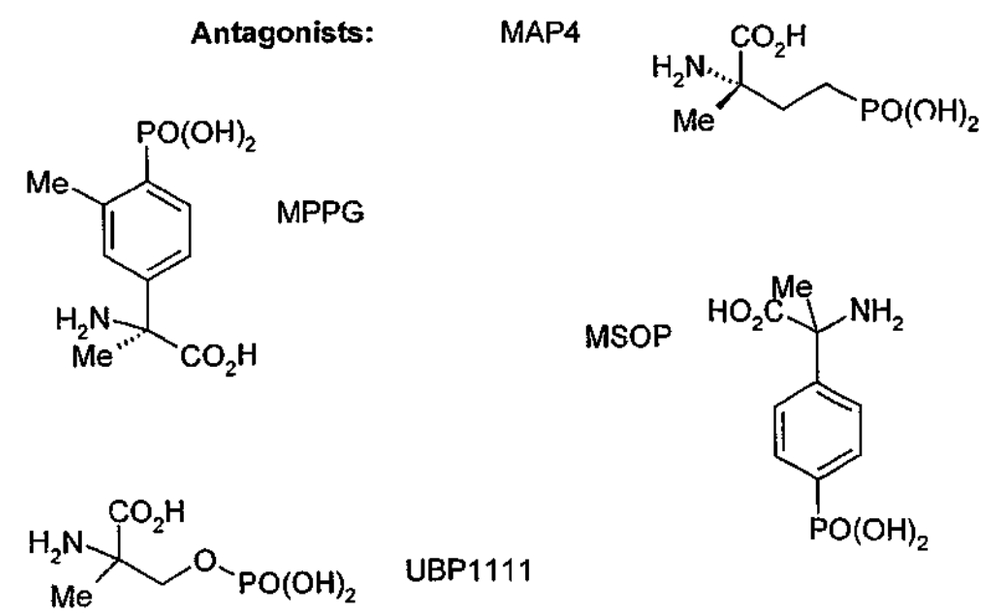


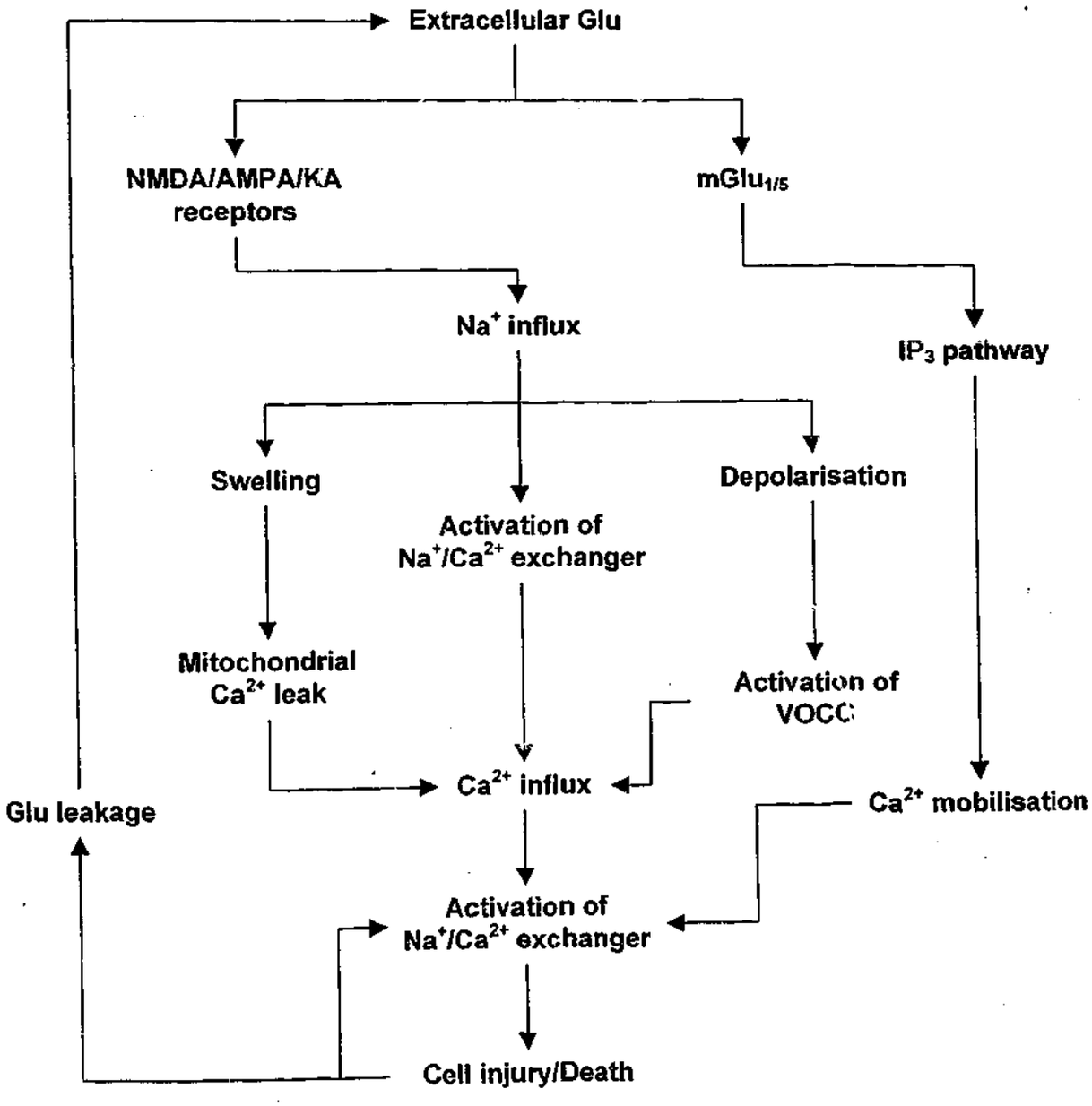
Figure 1.10 Chemical structures of group III mGlu receptor agonists and antagonists. Me: Methyl group.

(Faden et al., 1989). Excessive Glu exposure leading to neuronal injury has been termed *excitotoxicity* (Olney and Sharpe, 1969). Figure 1.11 maps some key pathways involved in our current understanding of excitotoxicity.

As circumventricular organs lie outside the blood brain barrier, they are vulnerable to dietary excitotoxins. Based on animal studies it is likely that infants with underdeveloped blood-brain barriers are particularly vulnerable to the dietary ingestion of Glu. For example, monosodium glutamate (MSG) is a common food additive, and animal studies have demonstrated neurodegeneration after oral administration of MSG (Olney and Sharpe, 1969) and convulsions after administration of many other EAAs (Johnston, 1973) that were more severe in immature, than mature animals. The best studied incidence of Glu receptor-mediated neurotoxicity after oral administration was in Canada in 1987, where patients experienced confusion, seizures, amnesia, coma and some died, after the ingestion of mussels contaminated with domoic acid (Bose et al., 1990; Teitelbaum et al., 1990). Pathological investigations revealed neurodegeneration of the hippocampus and amygdala, but no damage in the spinal cord or the brain stem.

Pioneering studies conducted by Olney and his colleagues demonstrated that Glu and its analogues induced neurodegeneration in areas with poorly developed blood brain barriers in immature rodents, with relatively no damage in adult counterparts unless high concentrations of Glu were used (Lucas and Newhouse, 1957; Olney, 1971). Early observations classified neurotoxicity mediated by Glu into to distinct types: that in which local necrosis occurred, commonly caused by Glu, NMDA and aspartate, and a second type which not only caused local necrosis but also sustained limbic seizures (including KA, QA, and AMPA) (Olney, 1983). From these early studies the selectivity of neuronal injury was also established, whereby non-neuronal cells were usually unaffected by Glu, unless extremely high concentrations were

Figure 1.11 Key pathways involved in the positive feed-back loop of excitotoxicity. VOCC: voltage operated calcium channels. Based upon Lipton and Rosenberg, 1994; Leist and Nicotera, 1998.



used (Olney, 1971). These studies also noted that different populations of neurones varied in their vulnerability to excitotoxins, possibly due to different Ca^{2+} buffering mechanisms and Glu receptor expression.

Once Glu was identified as a neurotoxin, many studies soon followed implicating endogenous Glu in the acute neuronal damage of various disease states such as ischaemia and epilepsy, and later the role of Glu in the slow, chronic neurodegenerative diseases. Amyotrophic lateral sclerosis (ALS) is characterised by selective loss of the lower motorneurones of the spinal cord and the upper motorneurones in the cerebral cortex (Mitosumoto et al., 1998), and patients suffering ALS eventually die from paralysis. Evidence to implicate Glu as a contributing factor in ALS have come from increased levels of Glu in the plasma and cerebrospinal fluid of patients suffering ALS, and there is some evidence to suggest the involvement of impaired Glu uptake systems (Lin et al., 1998; Mitosumoto et al., 1998).

A recognised role for Glu in various neurodegenerative diseases, such as Alzheimer's disease (AD), has become stronger in the past few years due to an increase in our understanding of Glu uptake systems. AD, a severe neurodegenerative disease, is characterised by severe memory loss and by the histological presence of plaques and neurofibrillary tangles (e.g. reviews: Jellinger, 1998; Iqbal et al., 2000; Shastry, 2001). A recent hypothesis has been advanced in AD implicating the defective functioning of GluTs, leading to a failure to clear excess Glu from the synaptic cleft (Masliah et al., 1996). Moreover, post-mortem analyses of brains from patients suffering AD demonstrate a marked increase in the expression of mRNA of the AMPA subunits Glu1-3 throughout the brain (Garcia-Ladona et al., 1994). Exposure of human spinal neurones to Glu results in morphological changes resembling neurofibrillary tangles (De Boni and McLachlan, 1985), and while in the presence of β-amyloid, a peptide

associated with plaques, Glu receptor-mediated toxicity is exacerbated (Gray and Patel, 1995). Based on observations with muscarinic receptors, Wurtman and colleagues undertook studies on the effect of mGlu receptor activation on amyloid precursor protein (APP) processing. They concluded that group I and II/III mGlu receptor agonists may enhance the conversion of full-length APP to nonamyloidogenic APPs in AD (Lee et al., 1995; Lee et al., 1997; Lee and Wurtman, 1997; Blanchard et al., 2002).

Huntington's chorea is a hereditary condition characterised by the selective degeneration of the spiny neurones of the neostriatum, which can be modelled in animals by intrastriatal injections of KA (Coyle et al., 1978). Cerebrospinal fluid of patients with Huntington's chorea contains elevated levels of Glu, and there is a depletion of the NMDA receptors in the striatum (Young et al., 1988). More recently, transgenic animal models of Huntington's chorea have demonstrated a decrease in the expression of AMPA and KA receptors, whereas no change in NMDA receptor expression is evident (Cha et al., 1998). Transgenic animal models of Huntington's chorea have a reduced sensitivity to KA and NMDA receptor-mediated toxicity (Hansson et al., 1999; Morton and Leavens, 2000) again indicating differential Glu receptor function and expression. Corticostriatal and thalamostriatal projections which innervate the GABAergic spiny neurones lost in Huntington's chorea, express mGlu receptors. While some evidence suggests that group I mGlu and NMDA receptor interactions contribute to selective striatal neuronal loss (Calabresi et al., 1999), further work is needed before a more complete understanding is reached regarding the role of mGlu receptors in Huntington's chorea.

Post-mortem tissue from patients with Parkinson's disease, a progressive neurodegenerative disease characterised by rigidity, tremor and bradykinesia, demonstrate a selective degeneration of neuromelanin-containing neurones, especially the nigral

dopaminergic neurones (Kastner et al., 1993; Stoessl, 1999). While evidence to link excitotoxicity to Parkinson's disease is limited, animals treated with 1-methyl-4-phenylpyridinium (MPP^+), to selectively damage dopaminergic neurones, are protected by the NMDA receptor antagonist MK-801 (Coyle and Puttfarken, 1993). Recently it has been shown that non-NMDA receptor antagonists are also effective at protecting against MPP^+-induced lesions in this model of Parkinson's disease (Merino et al., 1999; Klockgether et al., 1991), also implicating the non-NMDA receptors in this neurodegenerative disease. A number of studies have employed antagonists of group I mGlu receptors to inhibit chemically-induced parkinsonism demonstrating that such antagonists may prove useful in the management of Parkinson's disease, either alone or in conjunction with dopamine strategies (Kaatz and Albin, 1995; Abbott et al., 1997; Spooren et al., 2000; Awad et al., 2000; Marino et al., 2001; Popoli et al., 2001). Other approaches involve inhibiting excitatory input into the substantia nigra or the subthalamic nucleus using group II mGlu receptor agonists (Bradley et al., 2000; Dawson et al., 2000).

1.6.1.2 *Apoptosis versus necrosis*

Excitotoxicity generally involves two forms of neuronal cell death, *necrosis* and *apoptosis*. These two forms of cellular death appear to be dependent on the intensity of the insult (Lipton and Nicotera, 1998; Cheung et al., 1998b; Moldrich et al., 2000) and mitochondrial function (Ankarcrona et al., 1995). Necrosis is characterised by a rapid swelling of the cell, caused by a rapid influx of Na^+ ions which is followed by a passive, secondary influx of water and Cl^- ions. These osmotic changes result in the disruption of the internal and external cellular membranes, causing lysis of the cellular contents and thereby releasing cytoplasmic material, which is often toxic to neighbouring cells (Steller, 1995) and frequently

results in an inflammatory response *in vivo* (Kroemer et al., 1995). Apoptosis is a much slower process requiring energy and in some cases, protein synthesis (Wyllie et al., 1980; Dessi et al., 1994). Apoptosis is a normal physiological event that is involved in the removal of damaged cells in various tissues, ensuring a balance between cellular loss and proliferation throughout development and aging, and is essential for the correct functioning of most major organ systems, including the CNS (Kroemer et al., 1995; Oppenheim, 1991). Morphological changes induced by apoptosis include cell shrinkage, nuclear condensation, oligonucleosomal fragmentation of nuclear DNA to approximately 50-300 base pairs and neurite degeneration (Wyllie et al., 1984). The extrusion of intracellular organelles and nuclear fragments are rapidly digested by macrophages or by neighbouring cells *in vivo* and involves self-triggered phagocytosis, thereby avoiding inflammation (Savill et al., 1993; Kroemer et al., 1995; Philpott et al., 1996) and further toxic effects resulting from the leakage of potentially harmful substances (Clarke, 1990; Steller, 1995).

Studies of *in vitro* model systems have demonstrated that removal of Ca^{2+} from culture medium hinders apoptosis (Manev et al., 1989; Dessi et al., 1993), and the use of various Ca^{2+} channel antagonists can attenuate Glu receptor-mediated excitotoxicity involving apoptosis (Malcolm et al., 1996). For example, VOCC blockers are not protective against neurodegeneration that results from cellular death resembling necrosis (Weiss et al., 1990), but are highly protective against Glu-mediated apoptosis (Weiss et al., 1990; Pizzi et al., 1991). These findings have led to the hypothesis that an excessive influx of Ca^{2+} leads to elevation of free cytosolic Ca^{2+}, and that the resultant loss of Ca^{2+} homeostasis can subsequently influence numerous cellular mechanisms such as kinases and proteases, cellular metabolism and gene expression, ultimately resulting in apoptosis (Siesjo et al., 1989). An increase in free cytosolic Ca^{2+} can also result in the generation of toxic free radicals through activation of various

phospholipases and/or stimulate further release of Glu, increasing cellular damage by means of a positive feedback loop (see Figure 1.11; Choi et al., 1987). When prolonged stimulation of Glu receptors occurs, excessive amounts of Ca^{2+}-activated nitric oxide and superoxide free radicals are produced. Evidence for this mechanism has come from studies in which free radical scavengers have attenuated Glu receptor-mediated cellular damage (Whittemore et al., 1994; Simonian et al., 1996) and free radicals have been generated after excessive iGlu receptor stimulation (Savolainen et al., 1995; Coyle and Puttfarken, 1993).

Numerous genes have been identified as pro-apoptotic or "death" genes whereby their activation leads to apoptosis, or as anti-apoptotic genes which protect cells from various apoptotic stimuli (Dessi et al., 1994; Sastry and Rao, 2000). Genes thought to be involved in apoptosis were initially identified through studies in the nematode *Caenorhabditis elegans*, where homologues of many mammalian genes have been identified (Bargmann and Kaplan, 1998). Some examples of apoptotic genes include *c-myc*, caspases and BAX, whereas some anti-apoptotic genes include Bcl-2 and p35 (Vaux et al., 1988; Steller, 1995; Goldberg et al., 1997; Roth and D'Sa, 2001). A summary of the key morphological and biochemical changes involved in apoptosis and necrosis is presented in Table 1.8.

In a classical series of studies employing neuronal cultures Choi et al. (1987 & 1988) drew attention to the neurotoxic and pharmacological profile of Glu suggesting that Glu-induced cell death was occurring by necrosis. Later studies demonstrated lower concentrations of Glu could indeed also result in apoptosis (Ankarcrona et al., 1995; Portera-Cailliau et al., 1997; Nicotera et al., 1997; Cheung et al., 1998b). Necrosis can be triggered by both NMDA (Qin et al., 1996) and non-NMDA receptor activation (Frandsen et al., 1989; Larm et al., 1997; Giardina et al., 1998; John et al., 1999). Glu-induced toxicity is only partially attenuated by the selective NMDA receptor antagonist MK-801, with larger attenuation achieved by the co-

Table 1.8: Endpoints of neuronal death

		Apoptosis	Necrosis
Morphology	Cell body	Shrinkage	Swelling
	Neurophil	Fragmentation	Varicosities
	DNA	Discrete clumping	Diffuse shrinkage
		Cleavage into 200bp fragments	Generalised cleavage
	Mitochondria	Well preserved	Swelling
	Cell Membrane	Blebbing	Large blebbing imminent of lysis
Function	Membrane	Delayed permeability	Early permeability
	Enzymes	Preserved	Lost early
	Mitochondria	Preserved	Lost
Pharmacology		Protection by macromolecular synthesis inhibitors	Protection by macromolecular synthesis inhibitors

Adapted from Goldberg et al., 1997

addition of the non-NMDA receptor antagonist CNQX (Koh and Choi, 1991; Cheung et al., 1998b). While NMDA receptor agonists are understood to produce apoptotic cell death in primary neuronal cultures after short exposure times (5-30min) (Qin et al., 1996), a longer exposure is required for non-NMDA receptor agonists (12-48h) (Larm et al., 1997; John et al., 1999; Moldrich et al., 2000). Overstimulation of the KA receptor can result in apoptosis as demonstrated by studies conducted *in vitro* (Cheung et al., 1998a; Pollard et al., 1994; Moldrich et al., 2000) and *in vivo* (Le Gal La Salle, 1988; Gillardon et al., 1995), as can overstimulation of the AMPA receptor (Larm et al., 1997; John et al., 1999).

1.6.1.3 *In vivo versus in vitro models of excitotoxicity*

In vivo models of toxicity allow invaluable insights into the interaction of different cell types, such as the phagocytosis of apoptotic cells by macrophages (Fadok, 1999). However, identification of apoptotic cell bodies can be difficult *in vivo* due to the similarity in appearance of autophagotic vacuoles (Kerr, 1995) and the rapid clearance of dying cells by the immune system (Fadok, 1999). Due to the inability of many compounds to penetrate the blood-brain barrier, their direct application by injection or infusion can cause physical damage to the tissue resulting in necrotic cell death. In addition, the brain contains a high proportion of glial cells to neurones (10:1; Waxman, 1996), which serve as a protective system for the neurones, by secreting a variety of growth factors for example, and in so doing cloud the true extent of apoptosis induced.

Early *in vivo* studies used extremely high systemic doses of Glu to produce excitotoxicity in mature animals (total of 8 nmol/min over a period of one week) (McBean and Roberts, 1984). However, the problems inherent in such studies were compounded due to the presence of efficient GluT uptake systems (Vandenberg, 1998). Thus NMDA or KA were

routinely employed since neither of these compounds are readily taken up by GluTs, and by virtue of this are more potent neurotoxins *in vivo* than Glu itself (Johnston et al., 1979). In contrast, the full extent of excitotoxicity mediated by Glu and other agonists is evident using *in vitro* model systems (Choi et al., 1987). These systems are particularly favourable since they allow the researchers to control the milieu whereby GluTs can be readily blocked for example, allowing minute quantities of agonist to produce an insult (Frandsen and Schousboe, 1990). *In vitro* studies revealed that Glu was much more potent as a neurotoxin than what was initially understood from *in vivo* studies (Choi et al., 1987). Not only was the concentration of Glu required to cause cell death some 1000-fold less than that required to induce injury in the adult brain *in vivo* (Frandsen and Schousboe, 1990), the actual mode of cell death was more readily examinable (Ankarcrona et al., 1995). In addition, excitotoxicity was for the first time shown to be mediated by two forms of cell death, namely an initial necrotic cell death followed by a slower phase of cell death subsequently shown to be apoptosis (Ankarcrona et al., 1995; Portera-Cailliau et al., 1997; Cheung et al., 1998b). The translation of these discoveries *in vivo* then impacted on the understanding of trauma, stroke and epilepsy-induced toxicity, and subsequently, the approach used to develop treatments for these disorders. With current culture technology, various specific cell types can be isolated and readily examined, such as hippocampal cells, cerebellar granule and Purkinje cells, neocortical cells, glial cells and mesencephalic neurones. Alternatively, organotypic slice cultures of the hippocampus, striatum, and neocortices are popular models used to show regional toxicity and neuroprotection in a relatively intact system with conserved synaptic projections (Gahwiler et al., 1997). Therefore, while *in vivo* studies are essential for the full understanding of mechanisms in a physiological or pathological environment, *in vitro* studies have allowed the

molecular examination of cellular death at the cellular level in a controlled milieu with less confounding influences than are found in studies conducted in the intact CNS.

1.6.1.4 *mGlu receptors and excitotoxicity*

In the early 1990s, reports appeared documenting the protective and toxic effects of the early, non-selective mGlu receptor ligand, ACPD (McDonald and Schoepp, 1992; Siliprandi et al., 1992; Thomsen et al., 1993). Amongst these early reports, mGlu receptors were thought to be linked to NMDA receptor function; and, the role of IP_3 and PKC in contributing to increased intracellular Ca^{2+} and excitotoxicity was proposed (Favaron et al., 1990).

Agonists of group I mGlu receptors have been shown to act differentially during excitotoxicity depending on the experimental model or brain region under investigation. For example, activation of group I mGlu receptors has been shown to prevent Glu- and NMDA-induced toxicity in cultured cerebellar cells and hippocampal slices (Montoliu et al., 1997; Pizzi et al., 1993; Pizzi et al., 1996). In particular, it was discovered that the NMDA receptor subunit NR2C was crucial for group I mGlu receptor-mediated attenuation of Glu-induced excitotoxicity (Pizzi et al., 1999). Group I mGlu receptor activation of PKC induces phosphorylation of NMDA receptors containing NR2C subunits, which reduces the ability of glycine to enhance potentially toxic NMDA receptor-mediated responses. Group I mGlu receptor interactions with NMDA receptors may also occur via the scaffolding protein Homer, as discussed earlier. A second variable in the response of group I mGlu receptors is the presence of glia; for example, DHPG amplifies Glu-induced toxicity of cerebellar granule cells in the presence of glia (Nicoletti et al., 1999). Hence, it has been hypothesised that group I mGlu receptor activation causes the release of a neurotoxic factor from glia. In contrast, group

I mGlu receptor antagonists are generally thought to be neuroprotective in the presence of glia. For example, AIDA attenuates NMDA-induced excitotoxicity in some models (Cozzi et al., 1999), while MPEP appears to protect against β-amyloid peptide$_{(25\text{-}30)}$-toxicity in cultured cortical cells (Flor et al., 1999). Group I mGlu receptors have been shown to modulate *in vivo* and *in vitro* responses to post-traumatic neuronal injury (Mukhin et al., 1996) which was thought to involve the mGlu$_1$ subtype, particularly since specific mGlu$_1$ agonists exacerbate, and mGlu$_1$ antagonists reduce, these injuries (Allen et al., 2001; Faden et al., 2001). However, the mGlu$_5$ antagonist MPEP is also able to prevent toxicity during *in vitro* and *in vivo* trauma models (O'Leary et al., 2000; Bao et al., 2001; Movsesyan et al., 2001).

Agonists of group II mGlu receptors are thought to play a neuroprotective role during insult to cell cultures. Group II mGlu receptor agonists, L-CCG-I and the non-selective *t*-ACPD, protected against NMDA- and kainate-induced excitotoxicity, but also stimulated the production of inositol phosphates, suggesting this neuroprotection may result from activation of group I mGlu receptors (Bruno et al., 1994). DCG-IV however did not stimulate significant inositol phosphate production, but was neuroprotective, although the least potent of the three agonists tested. Furthermore, DCG-IV has been found to be neuroprotective against NMDA-induced toxicity in mixed cortical cultures (Kwak et al., 1996; Bruno et al., 1996). Part of this neuroprotective effect is thought to be due to the inhibition of presynaptic Ca^{2+} channels (Chavis et al., 1994) and inhibition of glutamate release (Ishida et al., 1993a,b). For example, decreasing intracellular cAMP levels via group II mGlu receptors could, in principle, decrease the 'open time' of high-threshold VOCCs and increase specific K^+ currents (e.g. K_{AHP}), which is responsible for after-hyperpolarisation (Bruno et al., 1995a; Cochilla and Alford, 1998). This action would decrease both neuronal excitability and postsynaptic Ca^{2+} influx, subsequently rendering the neurones less vulnerable to excitotoxic insult. A substantial contribution of this

neuroprotection appears to come from neurone-glia signalling (Bruno et al., 1997; Bruno et al., 1998; discussed in Chapter 2]. In contrast, LY354740 was not neuroprotective in NMDA or oxygen-glucose deprivation insults *in vitro* or *in vivo*, nor was it found to be neuroprotective in a rat model of ischaemia (Behrens et al., 1999). When tested at 10 μM, LY354740 was neuroprotective against NMDA insult, however Behrens et al. (1999) suggest that at this concentration such activity is probably due to non-selective group I mGlu receptor activation. A similar hypothesis exists for the group II agonist 4C3HPG, whereby the ability of this agonist to protect against NMDA insult was not reduced by a group II or group III mGlu receptor antagonist (Behrens et al., 1999). Clearly, more work is needed to delineate the roles of group II mGlu receptors in neuronal injury.

The group III mGlu receptor agonist, L-AP4, attenuated NMDA receptor-induced excitotoxicity in murine cerebellar granule cells, possibly by reversal of the glutamate uptake system (Lafon-Cazal et al., 1999). (+)-PPG is the active enantiomer of *R,S*-PPG, and has recently been shown to protect against NMDA-induced excitotoxicity *in vitro*, and striatal lesions and focal ischaemia *in vivo* (Flor et al., 1999a,b; Henrich-Noack et al., 1999). This *in vitro* neuroprotection was lost in cultures derived from $mGlu_4$-knockout mice, which suggests that the neuroprotective activity of (+)-PPG may be mediated by this receptor. Given the recent finding that astrocytes in culture express $mGlu_4$ (Besong et al., 2002), it is possible to speculate that $mGlu_4$-mediated neuroprotection may occur in a similar manner to that mediated by $mGlu_3$. Finally, an array of group III mGlu receptor agonists have been shown to induce neuroprotection in *in vitro* models of trauma (Faden et al., 1997).

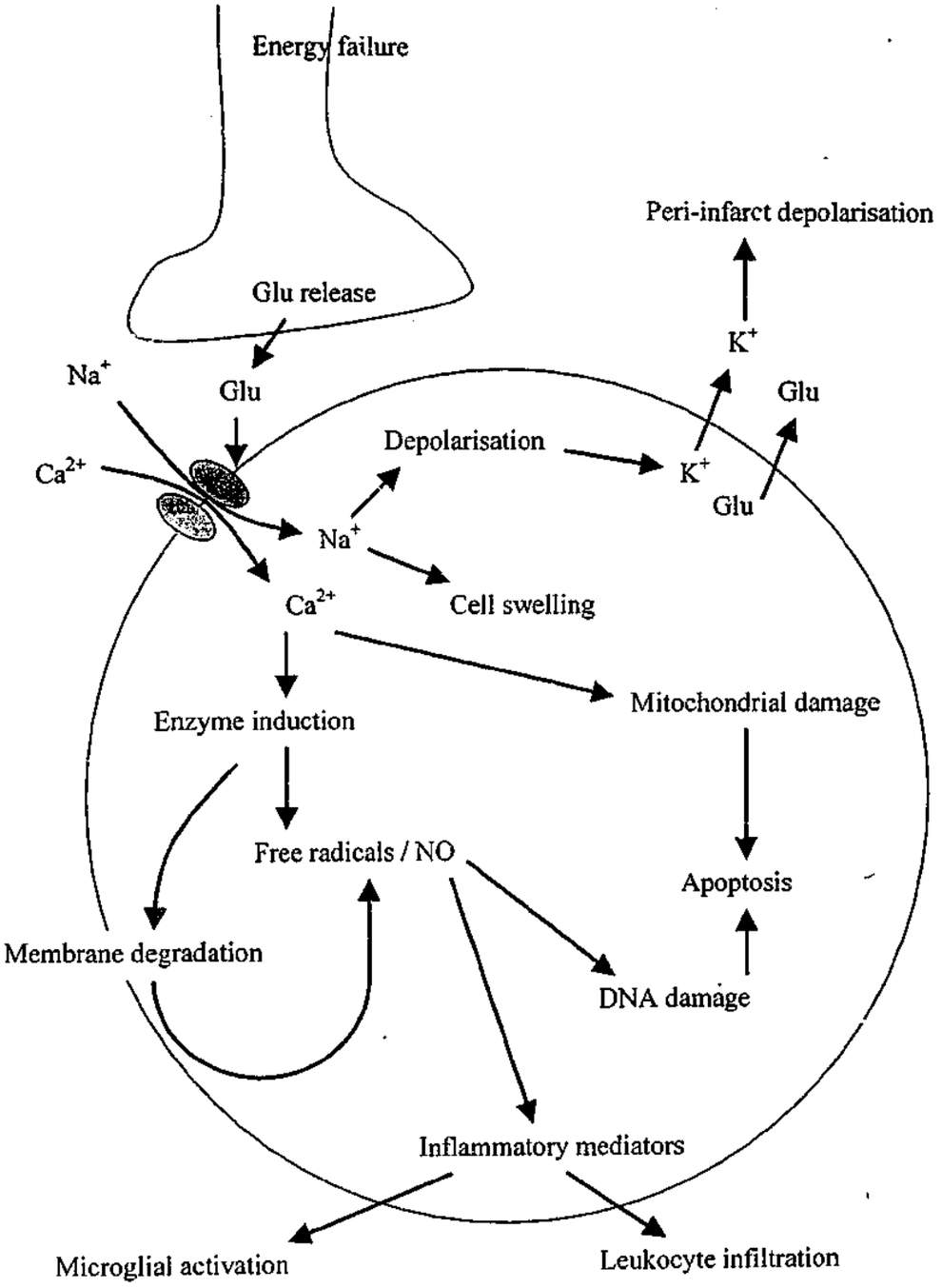
1.6.2 Ischaemic Stroke

Ischaemic stroke results from a transient or permanent reduction in cerebral blood flow that is restricted to the area surrounding a main cerebral artery. The reduction in blood flow is often caused by an occlusion which can be either an embolus or a local thrombosis. The brain has a relatively high consumption of oxygen and glucose, and depends almost entirely on local oxidative phosphorylation for energy production (Dirnagl et al., 1999). Therefore, a reduction in the supply of oxygen and glucose impairs the ability of the neurones to maintain ionic gradients, consequently, compromising the membrane potential and inducing neuronal and glial depolarisation. This depolarisation involves an imbalance of Ca^{2+} homeostasis which leads to Glu release and subsequent excitotoxicity (see Figure 1.12) (Choi, 1995). In studies of focal and global ischaemia, high concentrations of extracellular Glu were recovered from damaged brain regions (Faden et al., 1989), which were within the same concentration range as that found to be toxic *in vitro* (Choi et al., 1987). The most persuasive evidence to implicate Glu in ischaemic damage has come from studies employing Glu receptor antagonists, particularly the NMDA receptor antagonist MK-801, where there is an attenuation in post-ischaemic neuronal damage (Bond et al., 1999; Ikonomidou and Turski, 1995).

Neuronal necrosis was originally thought to be the primary cause of neurological deficit following induction of ischaemia in animal models (Degirolami et al., 1984). Accordingly, many early treatment strategies for the prevention of ischaemic damage aimed at countering the ion-influx cascades associated with necrosis. These strategies included the use of N-, P-, or L-type Ca^{2+} channel blockers, Na^{+} channel blockers, NMDA or AMPA receptor antagonists, and the reduction of reactive oxygen species or iron chelators (see Choi, 1995).

Further studies demonstrated that neuronal loss associated with ischaemia involves a predominantly necrotic core, centred around the site of cerebral blood flow occlusion, and an

Figure 1.12 Cellular pathways involved in ischaemia. Based upon Dirnagl et al., 1999.



outer apoptotic penumbra (where some perfusion is preserved) (Choi, 1995; Dirnagl et al., 1999). This apoptotic penumbra is thought to involve mitochondrial damage, DNA damage and/or selective gene expression and protein synthesis (see Dirnagl et al., 1999). Consequently, strategies for the treatment of ischaemia have broadened to include numerous targets including cytosolic enzymes, macromolecules, mitochondrial enzymes, protein synthesis, gene transcription, and other associated biochemical and morphological changes such as gliosis and inflammation. Since the use of iGlu receptor antagonists to prevent ischaemic damage at the clinical level has been limited, selective mGlu receptor agonists and antagonists have attracted considerable attention for their ability to indirectly modulate Glu neurotransmission and Ca^{2+} homeostasis (e.g. Bond et al., 1998; Pellegrini-Giampietro et al., 1999; Sabelhaus et al., 2000).

1.6.2.1 *mGlu receptors and stroke*

In general, oxygen-glucose deprivation for 60 min *in vitro* correlates with approximately 80% of the toxicity induced by 1 mM Glu over 24 h and is inhibited by NMDA, AMPA/KA (Small and Buchan, 1997; Gill and Lodge, 1997) and group I mGlu receptor antagonists (Maginn et al., 1995; Pellegrini-Giampietro et al., 1999). In the latter study, $mGlu_{1/5}$ agonists were not toxic when added alone to hippocampal cultures, but did exacerbate toxicity induced by oxygen-glucose deprivation, in agreement with other studies showing $mGlu_{1/5}$-mediated potentiation of NMDA toxicity in cortical wedges (Lehman and Neuman, 1996), cultured neocortical cells (Bruno et al., 1995b), hippocampal (Hu, John et al., 1996) and striatal slices (Pisani et al., 1997), and spinal cord ventral roots (Ugolini et al., 1997). Group II mGlu receptor agonists have been investigated in rat and gerbil models of focal and global ischaemia for their ability to reduce neuronal loss (Bond et al., 1998; Lam et al., 1998; Bond et al., 1999). As a result of these studies it appears that $mGlu_{2/3}$ agonists may induce neuroprotection by a

combination of actions, i.e. via presynaptic $mGlu_2$ receptors to inhibit glutamate release and possibly via glial $mGlu_3$ receptors to induce trophic factor release. However, this putative dual action does not appear to prevent acute neuronal loss, as occurs in the case of focal ischaemia. The group III mGlu receptor agonist *R,S*-PPG did not prevent neuronal loss in global cerebral ischaemia of the gerbil or rat, or focal cerebral ischaemia in the mouse (Sabelhaus et al., 2000), but significantly restored electrophysiological responses in hippocampal cultures after hypoxic/hypoglycaemic insult (Henrich-Noack et al., 2000).

Following 24 h of reperfusion in a gerbil model of global ischaemia $mGlu_2$ and $mGlu_4$ mRNA levels were significantly increased, while $mGlu_5$ mRNA expression levels were depressed (Rosdahl et al., 1994). Considering the neuronal localisation of the receptors, this study and those above suggested that the responses of infarct tissue and treatment with mGlu receptor ligands act to maintain the neuronal membrane potential below the threshold required for Glu release.

1.6.3 Epilepsy

The International League Against Epilepsy have categorised over 40 distinct epileptic conditions based upon characteristic symptoms and signs, seizure types, cause, age of onset and electroencephalogram (EEG) patterns (Table 1.9).

Generalised seizures encompass two of the most common forms of epileptic disorder: *absence* (petit mal) and *tonic-clonic* (grand mal) seizures. While there is enormous ambiguity surrounding the clinical definition and diagnosis of absence epilepsy they are primarily non-convulsive generalised seizures. Generally, absence seizures are characterised according to 2–4 Hz spike and wave discharges recorded on an EEG and can involve one or a number of associated components (Bladin, 1985). Absence seizures are predominant in children rather

Table 1.9 Epilepsy classification.
Compiled from Waxman, 1996; Bladin, 1985

Generalised	Primary	*Absence (petit mal)*	Childhood Absence Epilepsy Juvenile Absence Epilepsy Janz Myoclonic Epilepsy
		Tonic-Clonic (grand-mal)	Juvenile Myoclonic epilepsy
		Myoclonic	
		Atonic	
	Secondary	*Absence*	Lennox-Gastaut Syndrome (atypical absence)
		Simple Partial evolving to secondary tonic-clonic Complex Partial evolving to secondary tonic-clonic	
Partial (focal, local)	Simple (Jacksonian)	*Motor*	Frontal lobe epilepsy Benign Partial epilepsy
		Somatosensory	Parietal lobe epilepsy
		Special sensory	Occipital or temporal lobe epilepsy
		Autonomic	Temporal or Frontal lope epilepsy
		Psychic	Temporal lope epilepsy
	Complex	Simple Partial followed by impairment of consciousness and automatisms	Temporal or Frontal lope epilepsy
Generalised/Partial (Intermediate Petit-Mal) **Epilepsies associated with dementia or mental retardation** **Unclassified**			

than in adults, and are associated with emotional or biochemical precipitating factors (such as awakening or renal dysfunction; Berkovic, 1985). Brief lapses of consciousness during absence seizures can not only be detrimental to a child's learning potential and psyche, but can be life threatening. Tonic-clonic seizures are the most recognised seizure and involve repeated severe and synchronous convulsions (clonus) and extensions (tonus). Tonic-clonic seizures are often triggered by a visual stimulus (such as stroboscopic light) and can involve a preceding aura particular to the patient.

Partial seizures involve a focal group of neurones from which the seizure initiates (Waxman, 1996). If consciousness is not impaired during a partial seizure, it is considered a *simple partial* seizure; a *complex partial* seizure does involve impairment of consciousness. Simple partial seizures, involving a peripheral motor component, can be initiated by lesions from tumors of the motor cortex that spread to recruit regions of the homunculus cortex. Behaviour initiating from the temporal lobe, including facial automatisms, is associated with complex partial seizures and may involve tonic-clonic components.

Present clinically efficacious anti-epileptic drugs (AEDs) act via inducing prolonged inactivation of the Na^+ channel (e.g. phenytoin, carbamazepine, lamotrigine or valproate), blocking Ca^{2+} channel currents (e.g. ethosuximide) or by enhancing inhibitory GABAergic neurotransmission (e.g. diazepam, vigabatrin or tiagabine) (Meldrum and Chapman, 1999). Some AEDs act via a number of different mechanisms, which may also include antagonism of AMPA receptors (e.g. felbamate, phenobarbitone or topiramate). Most often, therapeutic regimes for epileptic patients will involve a change of first-line AEDs and/or add-on (adjunct) AEDs. Most AEDs are associated with adverse effects, some are mild such as sedation, dizziness and weight gain (e.g. topiramate, tiagabine and vigabatrin). Other adverse effects can be life threatening such as rashes leading to Stevens-Johnson syndrome (e.g. lamotrigine) or

aplastic anemia (e.g. felbamate) (Bougeois, 1998). Some epileptic patients are unresponsive to AED treatment. For this reason, research continues into safe and more effective AEDs.

1.6.3.1 mGlu Receptors and epilepsy

Excessive glutamatergic neurotransmission is understood to be one of the primary metabolic and pathological mechanisms behind the aetiology of numerous types of epilepsy (Chapman et al., 1996). A number of early studies showed that Glu, MSG and KA were capable of inducing epilepsy in animals that correlated with human symptoms (Johnston, 1973; Ben-Ari, 1985; Ben-Ari, 1981; Coyle et al., 1981; Meldrum et al., 1973). Since then, a number of functional changes in EAA neurotransmission have been reported in seizure-susceptible animals including: increased EAA-induced Ca^{2+} influx, altered EAA binding, enhanced Glu and Asp release, and modulation of Glu transporter expression and function (Meldrum et al., 1999).

Metabotropic Glu receptor ligands are relatively novel compared to benzodiazepines and Na^{+} channel inhibitors for example, consequently, the potential of mGlu ligands to attenuate epileptic seizures has not been full investigated, and at present there exists no mGlu receptor ligand in clinical use for the amelioration of epileptic seizures. Interest in iGlu receptor antagonists alone as potential anti-epileptic drugs (AEDs) increased with the discovery of the NMDA receptor antagonist MK-801 and the AMPA receptor antagonist GYKI 52466. Both these compounds possessed therapeutic potential in animal models of epilepsy (Chapman et al., 1991; Meldrum et al., 1992), however they failed early clinical toxicity trials and iGlu receptors have since lost popular favour as therapeutic targets for AEDs. However, iGlu receptors continue to be investigated to further understand the role of Glu in the aetiology of epilepsy. Reports that the "ACPD family" of mGlu receptor ligands could

inhibit glutamatergic neurotransmission in some brain regions initiated new interest in this target as a means of attenuating seizures. Especially since group II and III mGlu receptors are located presynaptically on glutamatergic nerve terminals and from previous experiments inhibit EPSPs.

Intra-amygdaloid injections of ACPD compounds inhibited the electrical kindling of the amygdala in rats and consequently inhibited the resulting lower seizure threshold-mediated clonic seizures (Attwell et al., 1995). Moreover, ACPD compounds decreased epileptiform activity in rat neocortical slices *in vitro* (Burke and Hablitz, 1994). However, *t*-ACPD and 1*S*,3*R*-ACPD in particular, continued to produce opposite effects in a variety of epileptic models (Schoepp et al., 1991a; Klitgaard and Laudrup, 1993; Mayat et al., 1994) reflecting the physiological duality noticed with these compounds in isolated cell culture and slice experiments. Thus clearly, more selective compounds would be required to identify the role which mGlu receptors played in epilepsy, especially as excellent animal models of epilepsy exist (Chapman and Meldrum, 1987).

Advances made with respect to the pharmacological distinction of mGlu receptors revealed that agonists of group I mGlu receptors could induce seizures (Tizzano et al., 1995), while antagonists could prevent seizures (Ghauri et al., 1996; Chapman et al., 1999; Chapman et al., 2000). The induction of seizures by group I mGlu receptors may involve synaptogenesis and/or protein synthesis (Merlin et al., 1998). In contrast, group II and III mGlu receptor agonists were thought to prevent seizures. A summary of the experimental anticonvulsant and proconvulsant profile of mGlu receptor ligands to date is found in Tables 1.10, 1.11 and 1.12.

While early, selective mGlu receptor ligands consistently proved efficacious in preventing seizures in animal models of epilepsy, few were potent when administered systemically. In fact, only the $mGlu_5$ antagonist MPEP had proved successful in inhibiting

Table 1.10. Anticonvulsant and proconvulsant activity of group I mGlu receptor ligands.

LIGANDS	ANTICONVULSANT ACTIVITY	PROCONVULSANT ACTIVITY
Agonists		
DHPG		• hyperexcitability/status epilepticus in rats[5] • limbic seizures in mice[4]
Antagonists		
S-4CPG	• Sound-induced seizures in mice[2] • DMCM and PTZ chemoconvulsant seizures in mice[2] • (no effect: electro-shock-induced seizures in mice)[2]	
MCPG[a]	• (no effect: sound-induced, PTZ-, DMCM-, electro-shock-induced seizures in mice)[2]	
AIDA	• Sound-induced seizures in mice[1] • SWD of absence seizures in lh/lh mice[1] • Sound-induced seizures in GEP rats[1]	• teeth chattering & head bobbing in GEP rats[1]
LY367385	• Sound-induced seizures in mice[1] • SWD of absence seizures in lh/lh mice[1] • Sound-induced seizures in GEP rats[1]	
SIB 1893	• Sound-induced seizures in mice (i.p.)[3] • CHPG or DHPG chemoconvulsant seizures in mice[3]	
MPEP	• Sound-induced seizures in mice (i.p.)[3] • CHPG or DHPG chemoconvulsant seizures in mice[3] • SWD of absence seizures in lh/lh mice[1]	

Anticonvulsant or proconvulsant activity was produced by intracerebroventricular or intracollicular injection unless otherwise indicated.
Abbreviations: DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate; GEP, genetically epilepsy prone; i.p., intraperitoneally; lh/lh, lethargic mice; PTZ, pentylenetetrazol; SWD, spike and wave discharge.
[a] also $mGlu_{2/3}$ antagonist
[1]Chapman et al., 1999 [2]Dalby and Thomsen, 1996 [3]Chapman et al., 2000 [4]Tizzano et al., 1995
[5]Camon et al., 1998

Table 1.11. Anticonvulsant and proconvulsant activity of group II mGlu receptor ligands.

LIGANDS	ANTICONVULSANT ACTIVITY	PROCONVULSANT ACTIVITY
Agonists		
L-CCG-I	• Sound-induced seizures in mice[1,5] • DHPG chemoconvulsant seizures[12] • (no effect: PTZ-, DMCM-, electro- shock-induced seizures in mice)[12]	• limbic seizures in mice[12]
DCG-IV	• KA chemoconvulsant seizures in rats[6] • GST in Am-kindled rats[7]	
1S,3R-ACPD[b]	• Sound-induced seizures in mice[1,5] • Seizure score in Am-kindled rats[2] • DMCM & NMDA chemoconvulsant seizures in mice[5]	• mild convulsions in rats[2] • limbic seizures in mice[12] • hyperexcitability/status epilepticus in rats[4]
1S,3S-ACPD	• GST in Am-kindled rats[8] • Epileptogenesis in Am-kindled rats[8] • Sound-induced seizures in GEP rats[10]	• mild convulsions in mice[1]
(S)-4C3HPG[a]	• DMCM & PTZ chemoconvulsant seizures in mice[5] • Electro-shock in mice[5] • Sound-induced seizures in GEP rats[9]	
2R,4R-APDC	• DHPG chemoconvulsant seizures in mice[2] • GST in Am-kindled rats[10]	• Decreased GST, Am-kindled rats[10]
LY345470	• PTZ, PIC & ACPD chemoconvulsant seizures in mice (i.p.)[3,11] • (no effect: NMDA chemoconvulsant seizures in mice)[3]	
LY379268	• ACPD chemoconvulsant seizures in mice (i.p.)[4]	
LY389795	• ACPD chemoconvulsant seizures in mice (i.p.)[4]	
Antagonists		
EGLU		• clonic seizures in mice[1]

Anticonvulsant or proconvulsant activity was produced by intracerebroventricular, intracollicular or intra-amygdaloid injection unless otherwise indicated.
Abbreviations: ACPD, (*1S,3R*)-ACPD; Am, amygdala; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate; GST, general seizure threshold; i.p., intraperitoneally; lh/lh, lethargic mice; PIC, picrotoxin; PTZ, pentylenetetrazol.
[a] also $mGlu_{1/5}$ antagonist [b] partial $mGlu_1$ agonist
[1]Meldrum et al., 1996 [2]Suzuki et al., 1996 [3]Klodzinska et al., 2000 [4]Monn et al., 1999 [5]Dalby and Thomsen, 1996 [6]Miyamoto et al., 1997 [7]Attwell et al., 1998 [8]Attwell et al., 1995 [9]Tang et al., 1997 [10]Attwell et al., 1998 [11]Monn et al., 1997 [12]Tizzano et al., 1995.

Table 1.12. Anticonvulsant and proconvulsant activity of group III mGlu receptor ligands.

LIGANDS	ANTICONVULSANT ACTIVITY	PROCONVULSANT ACTIVITY
Agonists		
R,S-PPG	• Sound-induced seizures in mice[1] • Sound-induced seizures in GEP rats[1] • Electroshock in mice[5]	
L-SOP	• (no effect: sound-induced seizures in mice)[3]	• clonic-tonic seizures in mice[3]
L-AP4	• Seizure score in Am-kindled rats[2] • Seziure score, SWD and GST in Am-kindled rats[4] • (no effect: sound-induced seizures in mice)[3]	• clonic-tonic seizures in mice[3]
Antagonists		
MCPA	• Sound-induced seizures in mice[3] • NMDA & DHPG chemoconvulsant seizures in mice[3]	• (no effect at high doses in mice)[3]
MAP4	• (no effect: sound-induced seizures in mice)[3]	• clonic-tonic seizures in mice[3]
MPPG	• (no effect: sound-induced seizures in mice)[3]	• clonic-tonic seizures in mice[3]

Anticonvulsant or proconvulsant activity was produced by intracerebroventricular, intracollicular or intra-amygdaloid injection.
Abbreviations: ACPD, (*1S*,3*R*)-ACPD; Am, amygdala; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate; GST, general seizure threshold; i.p., intraperitoneally; lh/lh, lethargic mice; PIC, picrotoxin; PTZ, pentylenetetrazol.
[1]Chapman et al., 1999 [2]Suzuki et al., 1996 [3]Ghauri et al., 1996 [4]Abdul-Ghani et al., 1997 [5]Gasparini et al., 1999

sound-induced seizures in mice when given intraperitoneally (Chapman et al., 2000). The lack of potent, systemically active mGlu receptor ligands has meant that their potential as AEDs is relatively unexploited.

Group I mGlu receptor antagonists have been found to reduce sound-induced clonic seizures in mice and seizures in genetically epilepsy prone rats (Chapman et al., 1999). This work supports earlier studies showing the anticonvulsant activity of the $mGlu_1$ antagonist/$mGlu_2$ agonist CHPG (Thomsen et al., 1994; Dalby and Thomsen, 1996) and the convulsant activity of the group I mGlu receptor agonist DHPG (Schoepp et al., 1994). Side effects resulting from benzodiazepine withdrawal in rodents include spontaneous seizures, increased muscle tone and a decrease in seizure threshold for convulsants (Suzuki et al., 1992; Mortensen et al., 1995). Recently it was discovered that the group I mGlu receptor antagonist (*S*)-4CPG suppressed the decrease in seizure threshold for the convulsant pentylenetetrazole following diazepam withdrawal in mice (Suzuki et al., 1999). Consequently, it appears that $mGlu_1$ not only contributes to the epileptic activity of tonic-clonic and absence seizures, but may also play a role in anxiety.

Previous approaches in the treatment of epilepsy have involved modulation of GABAergic neurotransmission or blocking of glutamatergic neurotransmission. The discovery that group II mGlu receptors act presynaptically to modulate glutamatergic and GABAergic transmission, and the development of selective agonists, has led to the investigation of the role of these receptors in rodent models of epilepsy. The $mGlu_1$ antagonist/$mGlu_2$ agonist CHPG was shown to reduce seizures induced by pentylenetetrazol, benzodiazepine ligands, and sound and electrical stimulation (Dalby and Thomsen, 1996). In the latter two models only L-CCG-I and 1*S*,3*R*-ACPD were found to be effective. DCG-IV was found to be effective in reducing electrical stimulated seizures in fully kindled rats (Attwell et al., 1998a) and reducing long-term

(but not short-term) effects of kainate-induced seizures (Miyamoto et al., 1997). Both of these studies failed to account for the NMDA receptor agonist activity of DCG-IV, hence it is possible that this non-selective activity masked the true anticonvulsant activity of this group II mGlu receptor agonist.

Activation of group III mGlu receptors presynaptically decreases the release of Glu and GABA. Intracerebroventricular injections of group III mGlu receptor agonists and antagonists have produced a variety of convulsant and anticonvulsant actions across a number of epileptic models. For example, the group III agonists L-AP4 and L-SOP and antagonists MAP4 and MPPG were found to be proconvulsant, while MCPA (a selective antagonist of L-AP4) was found to be anticonvulsant (Ghauri et al., 1996). Further experiments involving genetically epilepsy-prone rats showed that L-SOP caused prolonged anticonvulsant actions, despite inducing early acute seizures (Tang et al., 1997).

Apart from the pharmacological evidence for a role of mGlu receptors in seizures, changes in mGlu receptor expression have been found in human sufferers of epilepsy. Subtle differences appear to exist across epilepsy types (Glazier et al., 1997; Dietrich et al., 1999; Blumcke et al., 2000; Lie et al., 2000), with some studies reporting increases in $mGlu_1$, $mGlu_2$ and group III mGlu receptors while others have reported increases in only $mGlu_1$ and $mGlu_4$. Differences in group II and group III mGlu receptor expression have been found in human patients with mesial temporal lobe epilepsy (MTLE) (Tang and Lee, 2001). In this study receptor expression of $mGlu_{2/3}$ or $mGlu_8$ appeared confined to presynaptic terminals in the molecular layer or CA2, respectively. Furthermore, $mGlu_{2/3}$, $mGlu_4$ and $mGlu_8$ were found on astrocytes in the hippocampus of these MTLE patients which the authors concluded may relate to gliosis. In a similar series of experiments, immunoreactivity of group I mGlu receptors in these MTLE patients suggested that $mGlu_1$ and $mGlu_5$ may increase hippocampal

excitability through postsynaptic activation, and pre- and post-synaptic mechanisms, respectively (Tang et al., 2001a). Increased $mGlu_1$, but not $mGlu_5$, labelling was observed within the dentate gyrus molecular layer of chronic TLE patients, which correlated with the expression pattern found in animals with induced limbic seizures (Blumcke et al., 2000).

In animal models of epilepsy an upregulation of $mGlu_3$ and $mGlu_5$ has been observed in reactive astrocytes (Aronica et al., 2000; Ulas et al., 2000), while both $mGlu_8$ and $mGlu_1$ were found to be upregulated in the hippocampus of a rat pilocarpine model of status epilepticus (Tang et al., 2001b,c). Additionally, mice lacking $mGlu_7$ have been described as seizure-prone with an increase in excitability in cortical tissue (Sansig et al., 1999). No such trait has been reported in $mGlu_4$ or $mGlu_6$ knockout mice (Nakanishi et al., 1998).

1.6.3.2 *Epilepsy and toxicity*

Prolonged epileptic seizures produce a similar histopathological pattern to that of ischaemic damage (Meldrum, 1991), involving neuronal damage (Salcman et al., 1978; Meldrum et al., 1973; Ben-Ari, 1981; Olney, 1983; Auer and Siesjo, 1985; Lowenstein et al., 1992; Sutula, 2002) and astrogliosis (Khurgel et al., 1995; Morita et al., 1999). The glutamatergic system has been strongly implicated in neuronal damage as a result of seizure activity, since both pathologies are induced by DHPG, KA and NMDA administration (Olney et al., 1986; Olney et al., 1979; van Den Pol et al., 1996; Loscher, 1998), or can reduced by the appropriate Glu receptor antagonists (Clifford et al., 1989; Chapman et al., 1991; Chapman et al., 1991; Dingledine et al., 1990). In addition to pharmacological evidence for seizure-induced neurodegeneration, further direct evidence emerges from human epileptic conditions (Mathern et al., 1997; Mouritzen-Dam, 1980), particularly status epilepticus (Bengzon et al., 2002), tonic-

clonic seizures (Savic et al., 1998), temporal lobe epilepsy (Tang and Lee, 2001; Tang et al., 2001a; Mathern et al., 1995) and myoclonus (Takeda et al., 1988). Neuronal damage as a result of status epilepticus involves the alteration of apoptotic proteins such as Bcl-2 and caspases (Bengzon et al., 2002), similar to those involved in the apoptosis of stroke and trauma, and TLE induced by KA (Ben-Ari, 1985).

1.7. Thesis aims

In light of what is known regarding the ability of group II mGlu receptors to modulate glutamatergic neurotransmission, investigations into the extent and mode by which group II mGlu receptor ligands might promote neuroprotection and/or reduce the severity of seizures are important in developing therapeutic approaches for those disorders involving injury and epilepsy.

Thus the specific aims of this thesis are:

1. To evaluate the potential of group II mGlu receptor agonists to induce neuroprotection in neuronal cultures derived from the neostriatum, neocortex and cerebellar cortex.
2. To evaluate the potential of group II mGlu receptor agonists to protect against different modes of neurotoxicity, for example, apoptosis and necrosis.
3. To investigate the role of group II mGlu receptor function in astrocytes
4. To better understand the role of the second messenger, cAMP, in the function of group II mGlu receptors, and
5. To evaluate the potential of group II mGlu receptor agonists to reduce the severity and duration of seizures in animal models of human epilepsy.

CHAPTER 2

$mGLU_{2/3}$ AND *IN VITRO* NEURONAL INJURY

2.1. Introduction

Group II mGlu receptors have been shown to modulate neurotransmission (Hayashi et al., 1993; Flavin et al., 2000; Cartmell and Schoepp, 2000), and because of this role they have attracted considerable attention for their potential to reduce ionotropic Glu receptor-mediated excitotoxicity implicated in many neurological disorders (Nicoletti et al., 1996; Conn and Pin, 1997; Passani et al., 1997; Calabresi et al., 1999; Pellicciari and Costantino, 1999). The recent development of relatively selective $mGlu_{2/3}$ agonists (review: Schoepp et al., 1999) has allowed evaluation of this neuroprotective potential. Earlier studies into the neuroprotective potential of $mGlu_{2/3}$ employed the agonists 1-amino-cyclopentyl-1*S*,3*R*-dicarboxylate (1*S*,3*R*-ACPD), (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I) and (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV). However, these agonists possessed significant group I mGlu receptor or NMDA receptor activity, both of which have been shown to facilitate neurotoxicity. Despite such pharmacological profiles, these agonists were neuroprotective in a number of toxic conditions including excitotoxicity (Pizzi et al., 1993; Bruno et al., 1994; Bruno et al., 1995; Pizzi et al., 1996; Pizzi et al., 2000), oxygen-glucose deprivation (Small et al., 1996) and amyloidogenesis (Copani et al., 1995). Subsequently, Bruno and colleagues reported that neuroprotection in cultures containing neurones and glia required protein synthesis (Bruno et al., 1997), and that neurotrophic factors released by glia were most likely responsible for the neuroprotection observed during NMDA insult (Bruno et al., 1998a). More recently Kingston et al. (1999) demonstrated that the selective $mGlu_{2/3}$ agonists, including (1*S*,2*S*,5*R*,6*S*)-(+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), protected cortical cultures from excitatory amino acid- and staurosporine-induced toxicity, via a mechanism shown to be dependent upon the content of glia present. In contrast, Behrens

et al. (1999) claimed that LY354740 did not attenuate injury induced by NMDA or hypoxic-ischaemic insult *in vitro* or *in vivo*. Further discrepancies exist regarding the ability of selective $mGlu_{2/3}$ agonists to protect against neuronal injury in models of cerebral ischaemia (Kingston et al., 1999; Behrens et al., 1999; Bond et al., 1999). In many studies pure neuronal cultures have been abandoned, despite the role neurones play in early CNS development in the absence of elaborate glial networks, and even though neuronal $mGlu_{2/3}$ appear to inhibit neuronal glutamate release and be recruited during periods of high neurotransmitter release (Cartmell and Schoepp, 2000). Additionally, interpretation of data obtained in a mixed neuronal-glial milieu is likely to be confounded by glial "trophic" effects on neurones, whilst glia appear to have high levels of expression of mGlu receptors (Winder and Conn, 1996).

The present study is the first to comprehensively and systematically focus on the role of neurones in the neuroprotection reported to be induced by the activation of $mGlu_{2/3}$. Essentially glial-free neuronal cultures from three brain regions were employed and the role of multiple factors that could possibly influence neuronal responses to $mGlu_{2/3}$ agonists were investigated. These factors include the development and type of neurones, mode of insult induced, degree of insult, activity of $mGlu_{2/3}$ agonists, concentration of agonists and mode of agonist treatment. Varying modes of insult were investigated, including the free radical generator hydrogen peroxide, the non-selective protein kinase inhibitor staurosporine, low K^+-induced apoptosis and the excitatory amino acids (EAA) NMDA, AMPA and KA. Traditional agonists L-CCG-I, DCG-IV and *N*-acetylaspartylglutamate (NAAG) were evaluated with particular focus given to the selective agonist 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylic acid (2*R*,4*R*-APDC). A preliminary

account of some of these findings was presented at the 3rd International Meeting on Metabotropic Glutamate Receptors (Moldrich and Beart, 1999).

2.2. Methods

All animal experiments in this chapter were performed according to the Animal Ethics Guidelines of Monash University under animal ethics approval Pharmacology 1999/09. A full list of materials used appears in Appendix IV. Full culture methods appear in Appendices VI-VII.

2.2.1. *Cortical and striatal neuronal cultures*

Cells from the cerebral neocortex and striatum were similarly cultured from Swiss white embryonic mice (day 15-16) as previously described (Cheung et al., 1998b). The cortices and striatum were dissected in Hank's Balanced Salt Solution (HBSS; Appendix III) containing bovine serum albumin (3 mg/ml) and 1.2 mM $MgSO_4$. Tissue was digested with trypsin (0.2 mg/ml) and DNase (80 μg/ml) at 37°C for 5 min. Digestion was terminated by the addition of trypsin inhibitor (0.52 mg/ml) and the cells were triturated and suspended in Neurobasal medium (Appendix I) containing B27 (Appendix II), penicillin (100 U/ml) and streptomycin (100 μg/ml), 0.5 mM glutamine and 10% fetal calf serum. Cells were seeded in Nunc mutliwell plates at 0.12×10^6 or 0.3×10^6 cells/well for pharmacological (96-well plates) or morphological/cAMP assay (24-well plates) experiments, respectively. After 24 h *in vitro*, the medium was replaced with serum-free Neurobasal medium containing B27 supplement, and cultures were maintained in a humidified CO_2 incubator (5% CO_2, 8.5% O_2; 37°C) for up to 13 days *in vitro* (div). Partial medium changes were performed each 4-5 div.

2.2.2. *Cerebellar granule cell culture*

Cerebella were dissected from Swiss white mice pups (postnatal day 7) and cerebellar granule cells (CGC) cultured as described above with some modifications (c.f. Cheung et al., 1998a). Cerebellar tissue was digested in HBSS containing BSA (3 mg/ml) and 1.2 mM $MgSO_4$ with trypsin (0.2 mg/ml) and DNase (160 μg/ml) for 30 min at 37°C, and the digestion was terminated by addition of trypsin inhibitor (0.52 mg/ml). Cells were triturated and seeded in 24-well plates at a density of 0.4×10^6 cells/well for all experiments. After 24 h *in vitro*, the medium was replaced with Neurobasal medium containing B27, 25.4 mM KCl, penicillin/streptomycin and 10 μM aphidicolin. Cells were maintained in a humidified CO_2 incubator and a medium change was performed every 3 days after 6 div.

2.2.3. *Immunocytochemistry*

Immunocytochemistry was performed at 6, 9 and 13 div. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cultures were quenched with Stable Peroxide Substrate and non-specific binding blocked with 10% normal goat serum (NGS) and 0.1% Triton X-100 in Tris-buffered saline (TBS) for 1 h at 4°C. Cells were incubated overnight at 4°C with either anti-microtubule associated protein-2 (MAP-2; 1:500 dilution) or anti-glial-fibrillary acidic protein (GFAP; 1:200). Secondary antibodies were incubated for 3 h at 25°C with 2% NGS, 0.1% Triton X-100 in TBS (1:200; anti-rabbit for GFAP and $mGlu_{2/3}$, anti-mouse for MAP-2). Immunoreactivity was developed with 3,3'-diaminobenzidine (DAB, 0.5 mg/ml) in Stable Peroxide Substrate and visualised using brightfield microscopy. To assess the purity of cultures counts were made of cells immunoreactive for GFAP across nine fields from three independent cultures.

2.2.4 *Cell viability assay*

Cultures were exposed to toxic insults at 6, 9 and 13 div for either 4, 24 or 48 h in a humidified CO_2 incubator at 37°C. Cortical and striatal neurones were exposed to 60-110 μM H_2O_2, 1 μM staurosporine, 100 μM AMPA and 100 μM KA in antioxidant-free N2-supplemented Neurobasal medium (Cheung et al., 1998b; Appendix III). This latter vehicle medium contains 5.4 mM KCl, and is not toxic to cortical or striatal neurones, but as well documented, induces apoptosis in CGC cultures (Ikeuchi et al., 1998). Hence, this medium served as a toxic insult for CGCs. Dulbecco's Modified Essential Medium (DMEM) served as the vehicle for the NMDA (70 μM) insult in cortical and striatal neurones. All treatments were incubated in a humidified CO_2 incubator to allow solutions to reach 37°C and pH of the cultures before addition to the cells. Group II mGlu receptor agonists evaluated included L-CCG-I (1-300 μM), DCG-IV (1-300 μM), NAAG (1-300 μM) or 2*R*,4*R*-APDC (1-100 μM). Agonists were co-incubated with the insult for the complete timecourse, additional experiments were carried out in the presence of MK-801 (10 μM) and AIDA (10 μM) for DCG-IV and L-CCG-I respectively. Pre-incubation experiments were performed with 2*R*,4*R*-APDC whereby cultures were pre-treated with the agonist for 2 h prior to the addition of the insult. Acute toxicity experiments were performed with 150 μM H_2O_2, 10 μM staurosporine, 300 μM NMDA, 1000 μM AMPA and 1000 μM KA for 30 min with 2*R*,4*R*-APDC, followed by a 24 h incubation with vehicle or 2*R*,4*R*-APDC only. Cultures were routinely exposed to vehicle medium only (100% cell viability control) and 0.1% Triton X-100 (0% cell viability control). Cellular viability was examined by phase-contrast microscopy and by the measurement of formazan produced by the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells (Cheung et al., 1998b). MTT (5 mg/ml) was prepared in RPMI 1640 growth medium and was

incubated with the cells at 37°C for 30 min following the injury timecourse. The resulting formazan product was dissolved in 20% sodium dodecyl sulfate and 40% dimethylformamide. The optical density was measured at 570 nm using a microplate reader.

2.2.5 *Measurement of cAMP*

The cellular content of cAMP in cortical, striatal and cerebellar granular neurones (9-12 div, 24-well plates) was measured following treatment with 10 μM forskolin and/or 2*R*,4*R*-APDC (1-30 μM) in the presence or absence of the $mGlu_{2/3}$ antagonist (2*S*,4*S*)-2-amino-4-(4,4-diphenylbut-1-yl)pentane-1,5-dioic acid (LY307452; 100 μM; Schoepp et al., 1999). Cultures were washed, and incubated for 5 min at 37°C in HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% (v/v) glucose, 20 mM HEPES; pH 7.2) containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and treatments. Ca^{2+} was omitted from the incubation buffer to avoid inhibition of AC (Lui et al., 1998). Following aspiration of the buffer, cellular cAMP was extracted by scraping the well in the presence of 1.75% (v/v) ice-cold $HClO_4$. The final extraction volume was made up to 250 μl with 1.2 M K_2CO_3. Following centrifugation of the extract at 13000 × g, aliquots of the supernatant were assayed according to the commercially available Biotrak [^{3}H]cAMP assay kit.

2.2.6. *Statistics*

For the cellular viability assay, optical density values were standardised against the vehicle medium only (100%) or 0.1% Triton X-100 (0%) which causes rapid lysis of cells. The mean ± S.E.M. of the treatment was calculated from 3-4 wells per culture, and across 2 independent cultures. Treatment groups were analysed using one-way analysis of variance (ANOVA) and significant differences identified where $P < 0.05$.

2.3. Results

2.3.1. *Characterisation of cortical and striatal cultures*

Morphological examination by phase-contrast microscopy of each cell culture was made at 6, 9 and 13 div. At 6 div cortical cultures consisted of an extensive neurite network spanning clusters of neuronal cell bodies. This network was less extensive in striatal and cerebellar cultures at this time point. At 9 div, an extensive and evenly distributed neuronal monolayer was observed for CGCs. At 13 div, a healthy neuronal monolayer was observed in the striatal cultures, but at which time cortical cultures showed an increase in background cell death. Counts of cells immunoreactive for GFAP revealed that at any div cortical, striatal and cerebellar cultures contained a maximum (mean ± S.E.M.) of 5.5 ± 0.8%, 6.6 ± 1.3% (13 div) and 1.5 ± 0.8% glia respectively (Figure 2.1, Table 2.1).

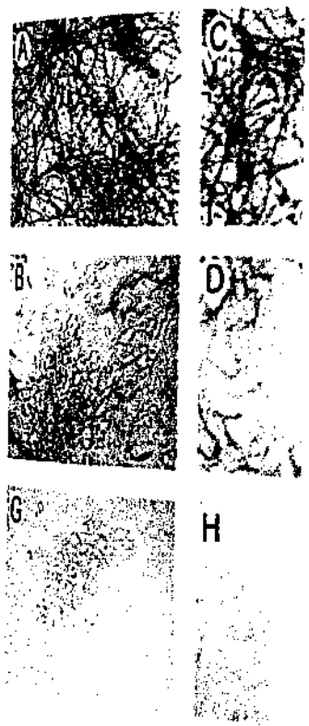
2.3.2. *Group II mGlu receptor agonists and cellular viability after various neurotoxic insults*

2.3.2.1. *Co-incubation with group II mGlu receptor agonists*

Graded reductions in cellular viability were produced by exposure of the cultures to varying toxic insults for either 4, 24 or 48 h. In both cortical and striatal cultures, EAA-induced toxicity was not substantial by 4 h, hence measurements of cellular viability were only made at 24 and 48 h. In N2-supplemented Neurobasal medium, NMDA (300 μM) caused a maximum of 30% toxicity at 48 h (not shown). Hence, DMEM was employed as the vehicle resulting in toxicity of >50% with 70 μM NMDA.

In cortical neuronal cultures, concentrations of 70-100 μM H_2O_2 were employed at 6, 9 and 13 div to induce a decrease in cellular viability of approximately 15-25%, 50-60% or 70-90% from control at 4, 24 or 48 h respectively. 2*R*,4*R*-APDC (1-100 μM), DCG-IV (1-300 μM), L-CCG-I (1-300 μM), and NAAG (1-300 μM) failed to

Figure 2.1 Immunocytochemistry of cortical, striatal and cerebellar neuronal cultures. Bright-field photomicrographs of cortical (A & B, 9 div), striatal (C & D, 13 div) and cerebellar granular neurones (E & F, 9 div) showing labelling of anti-MAP-2 (A, C, E) and anti-GFAP (B, D, F). Note the comparatively prolific labelling of the cultures with anti-MAP-2 compared to anti-GFAP. Non-immune controls for anti-MAP-2 (G), anti-GFAP (H) are shown for cortical neurones but are representative of all cultures. Scale bar = 75 μm.



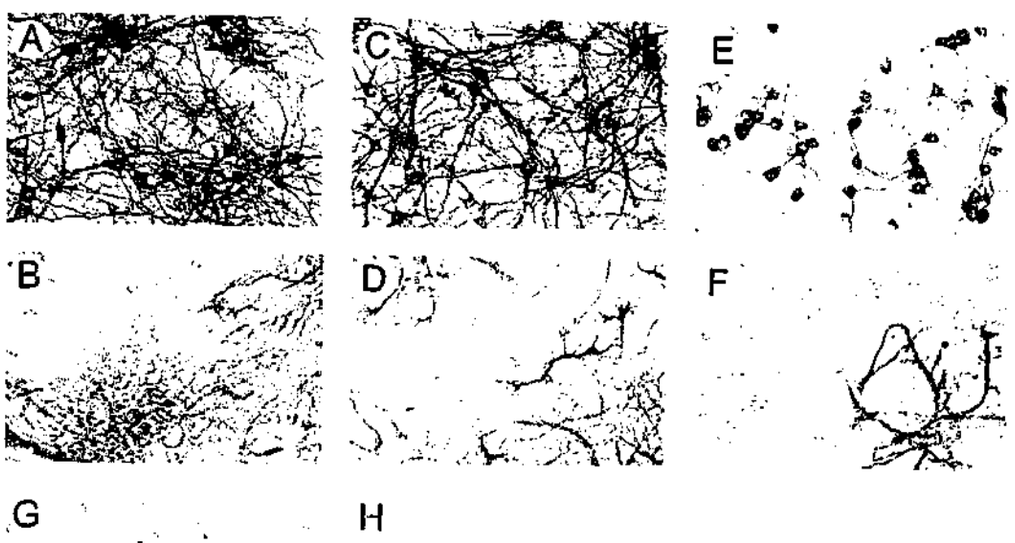

A
C
E
B
D
F
G
H

Table 2.1 Quantification of glial content in neuronal cultures as determined by anti-GFAP immunocytochemistry

	% Positive anti-GFAP labelled cells		
day *in vitro*	cortical cultures	striatal cultures	CGC cultures
6	4.1 ± 0.7	3.3 ± 0.2	1.8 ± 0.3
9	4.4 ± 0.5	4.8 ± 0.6	1.1 ± 0.6
13	5.5 ± 0.8	6.7 ± 1.3	1.5 ± 0.8

Mean ± S.E.M. of 9 fields from 3 independent cultures.

attenuate the decrease in cellular viability produced by H_2O_2 (Figures 2.2-2.5 and Table 2.3B). Staurosporine (1 μM) induced a more stable decrease in cellular viability at each div, with cultures exhibiting approximately 90%, 30% and 10% cellular viability at 4, 24 and 48 h, respectively. 2*R*,4*R*-APDC (1-100 μM), DCG-IV (1-300 μM), L-CCG-I (1-300 μM), and NAAG (1-300 μM) failed to attenuate the decrease in cellular viability produced by staurosporine (Figures 2.2-2.5 and Table 2.3B). Both KA (100 μM) and AMPA (100 μM) demonstrated similar excitotoxic profiles, such that each EAA caused a decrease in cellular viability to approximately 50% and 30% of control at 24 and 48 h, respectively. NMDA (70 μM) generally produced a greater decrease in cellular viability, ranging from 50% at 24 h to 20-25% at 48 h. None of the $mGlu_{2/3}$ agonists attenuated the EAA-induced decrease in cellular viability (Figure 2.2-2.5 and Table 2.3B).

In striatal neuronal cultures, concentrations of 60-110 μM H_2O_2 over 6, 9 and 13 div were employed to induce a decrease in cellular viability similar to that of cortical neurones. 2*R*,4*R*-APDC (1-100 μM), DCG-IV (1-300 μM), L-CCG-I (1-300 μM), and NAAG (1-300 μM) failed to attenuate this decrease in cellular viability (Figures 2.2-2.5 and Table 2.3B). In contrast to cortical neurones, staurosporine (1 μM) only produced a decrease in cellular viability to approximately 90%, 50% and 30% of control at 4, 24 and 48 h, respectively. Again the agonists failed to attenuate this injury (Figures 2.2-2.5 and Table 2.3B). NMDA (70 μM), KA (100 μM) and AMPA (100 μM) demonstrated similar excitotoxic profiles in striatal neurones, each EAA caused a decrease in cellular viability that was substantially less than that induced in cortical neurones (approximately 70% and 50% of control at 24 and 48 h, respectively). Group II mGlu receptor agonists also failed to attenuate EAA-induced injury in striatal neurones also (Figures 2.2-2.5 and Table 2.3B).

N2-supplemented medium containing low K^+ (5.4 mM KCl) produced a decrease in the viability of cerebellar granule neurones to approximately 90%, 30% and 10% of control after 4, 24 and 48 h, respectively. 2*R*,4*R*-APDC (1-100 μM), DCG-IV (1-300 μM), L-CCG-I (1-300 μM) and NAAG (1-300 μM) failed to attenuate this decrease in cellular viability at either 6, 9 or 13 div (Table 2.2 and Table 2.3B).

Experiments in all cultures with the agonists DCG-IV and L-CCG-I were repeated in the presence of the NMDA receptor antagonist (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine (MK-801, 10 μM) or the $mGlu_1$ antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA, 10 μM). Some minor attenuation of NMDA-induced toxicity was noticed in those experiments with DCG-IV and MK-801, but this neuroprotection was attributable to the activity of MK-801 (not shown). Similarly, where DCG-IV concentrations of 100 μM or more were used, a decrease in cellular viability in addition to that induced by the insult under investigation was noticed, which was abolished by co-incubation with MK-801 (not shown). With this exception of DCG-IV, none of the agonists affected the cellular viability of the cultures when tested up to the maximum concentration used in the absence of insult.

2.3.2.2. *Pre-incubation and treatment of acute toxicity with 2R,4R-APDC*

Experiments involving pre-incubation of 2*R*,4*R*-APDC (1-30 μM) were carried out at either 9 div for cortical and cerebellar cultures or 13 div for striatal cultures, at which stage healthy neuronal monolayers were prevalent. 2*R*,4*R*-APDC was incubated with the cultures in the presence of the experimental vehicle mediums or growth medium (in the case of cerebellar cultures) 2 h prior to a medium change which included 2*R*,4*R*-APDC and the insult (as above). Following 24 h exposure to this latter medium, 2*R*,4*R*-APDC did not

attenuate the decrease in cellular viability induced by the insults (Table 2.3A). Additionally, 2*R*,4*R*-APDC (1-30 μM) failed to attenuate toxicity induced by 30 min exposure to 150 μM H_2O_2, 10 μM staurosporine, 300 μM NMDA, 1000 μM KA or 1000 μM AMPA (Table 2.3C).

2.3.3. *Cell morphology*

Each insult compromised the morphological integrity of the neuronal monolayer in each culture, with a pattern of neuronal injury which was characteristically time-dependent (Figure 2.6). Each insult, in general, caused the degradation of neurites and the breakdown of neuronal bodies; H_2O_2 also caused some cellular swelling indicative of necrosis (Cheung et al., 2000). In contrast, both staurosporine and AMPA in cortical and striatal cultures, and low K^+ in CGCs, caused cellular shrinkage, neurite blebbing and formation of pyknotic bodies consistent with apoptotic-like neuronal injury (Cheung et al., 1998b; Moldrich et al., 2000; Cheung et al., 2000). In all experiments described above $mGlu_{2/3}$ agonists, on the basis of the assessment of the morphological features of the relevant patterns of injury arising from any insult, failed to protect cortical, striatal and CGC neurones from the pathological consequences of neurotoxicity.

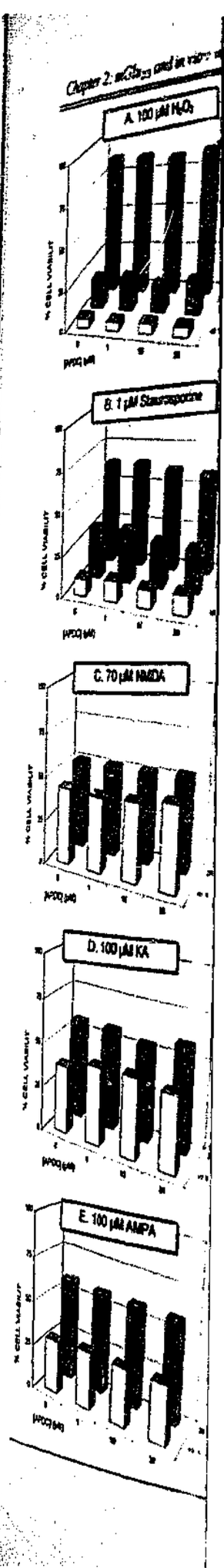
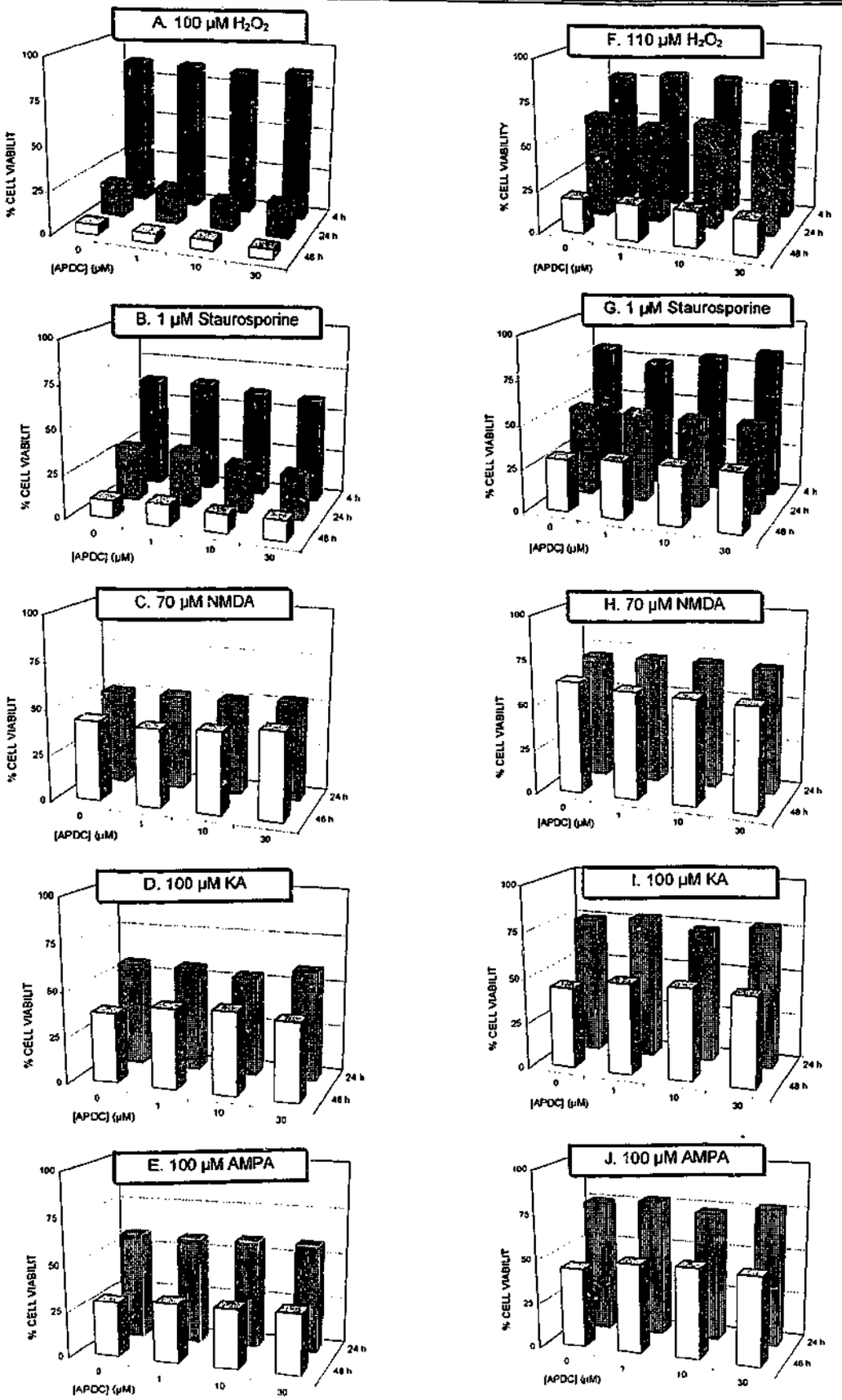
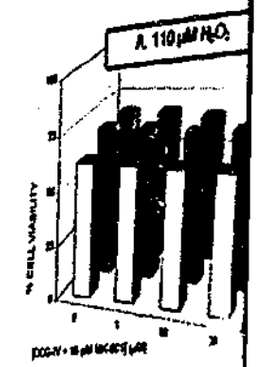
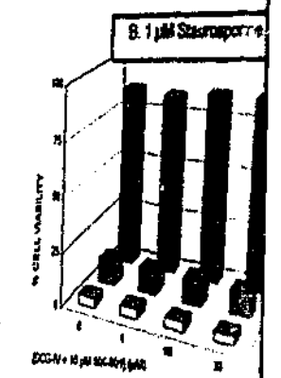


Figure 2.2 2*R*,4*R*-APDC fails to attenuate the decrease in cell viability induced by various insults. Bar graphs represent results obtained with cortical neurones (A-E, 9 div shown) or striatal neurones (F-J, 13 div shown) at each div. For each insult and time-point 2*R*,4*R*-APDC treatment (1-30 μM) was not significant from that of insult only (One-way ANOVA, $P > 0.05$, $n = 6$-8).


A. 100 µM H_2O_2
F. 110 µM H_2O_2
B. 1 µM Staurosporine
G. 1 µM Staurosporine
C. 70 µM NMDA
H. 70 µM NMDA
D. 100 µM KA
I. 100 µM KA
E. 100 µM AMPA
J. 100 µM AMPA
% CELL VIABILIT
[APDC] (µM)
4 h
24 h
48 h





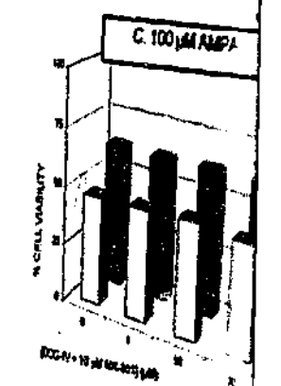
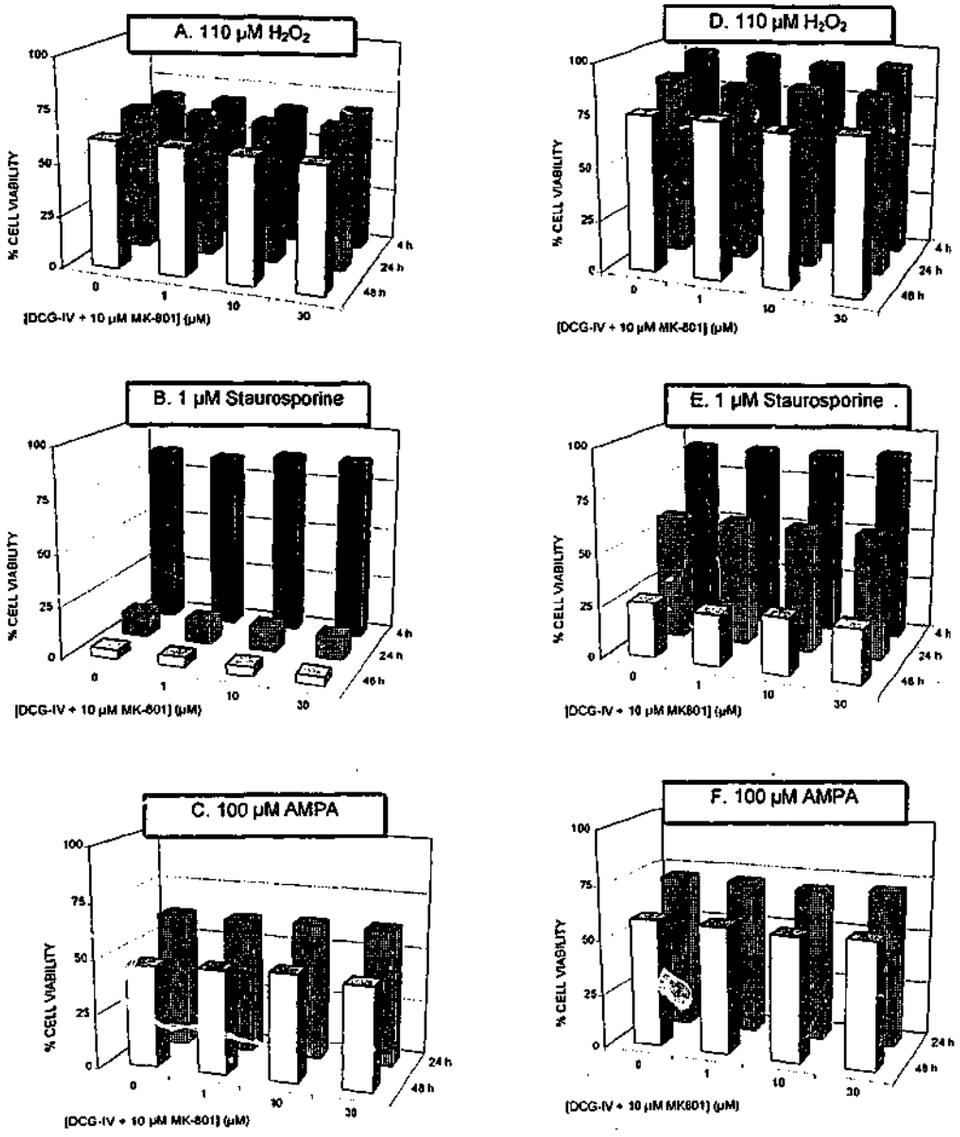


Figure 2.3 DCG-IV, in the presence of the NMDA receptor antagonist MK-801 (10 μM), fails to attenuate the decrease in cell viability induced by various insults. Bar graphs represent results obtained with cortical neurones (A-C, 9 div shown) or striatal neurones (D-F, 13 div shown) at each div. For each insult and time-point DCG-IV (1-30 μM) was not significant from that of insult in the presence of MK-801 (One-way ANOVA, $P > 0.05$, n = 6-8).


A. 110 µM H_2O_2
D. 110 µM H_2O_2
B. 1 µM Staurosporine
E. 1 µM Staurosporine
C. 100 µM AMPA
F. 100 µM AMPA
% CELL VIABILITY
[DCG-IV + 10 µM MK-801] (µM)
4 h
24 h
48 h

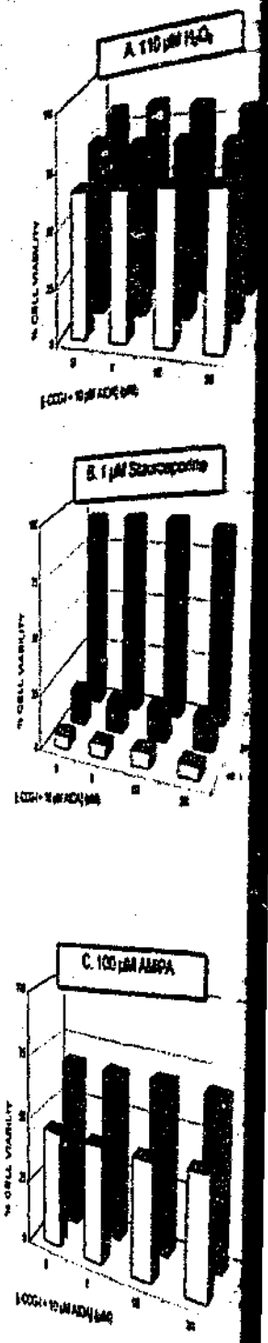
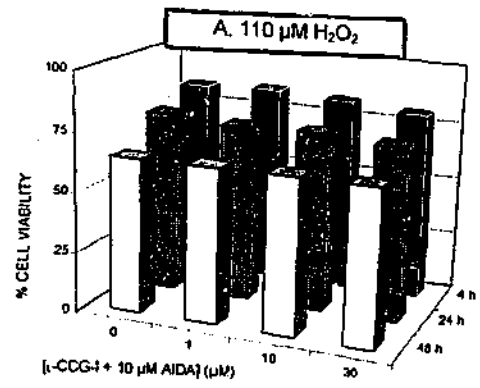
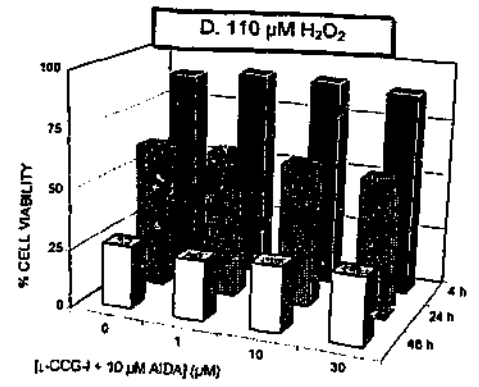
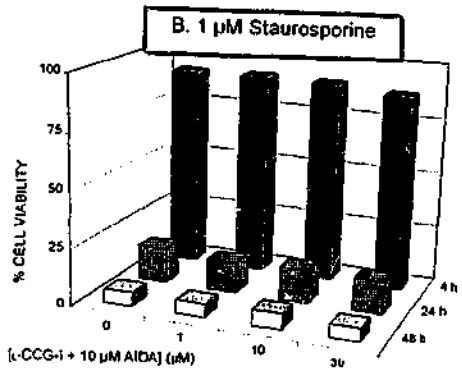
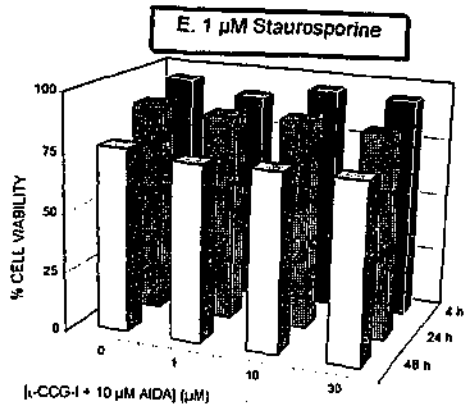


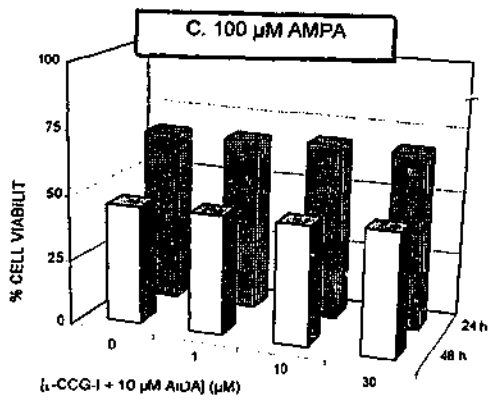
Figure 2.4 L-CCG-I, in the presence of the mGlu$_{1/5}$ antagonist AIDA (10 μM), fails to attenuate the decrease in cell viability induced by various insults. Bar graphs represent results obtained with cortical neurones (A-C, 9 div shown) or striatal neurones (D-F, 13 div shown) at each div. For each insult and time-point L-CCG-I (1-30 μM) was not significant from that of insult in the presence of AIDA (One-way ANOVA, $P > 0.05$, $n = 6$-8).

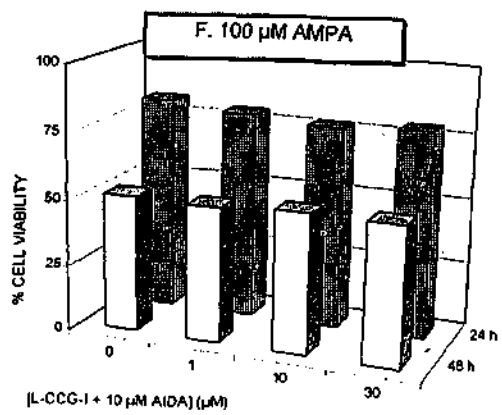

A. 110 µM H_2O_2
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
4 h
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)

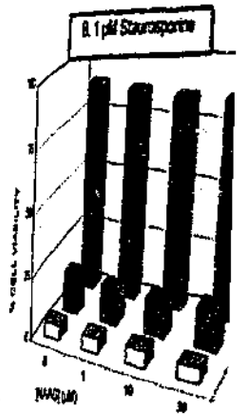

D. 110 µM H_2O_2
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
4 h
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)


B. 1 µM Staurosporine
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
4 h
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)


E. 1 µM Staurosporine
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
4 h
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)


C. 100 µM AMPA
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)


F. 100 µM AMPA
% CELL VIABILITY
100
75
50
25
0
0
1
10
30
24 h
48 h
[L-CCG-I + 10 µM AIDA] (µM)



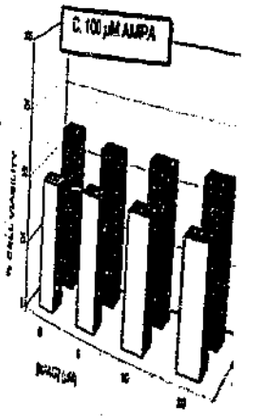


Figure 2.5 NAAG fails to attenuate the decrease in cell viability induced by various insults. Bar graphs represent results obtained with cortical neurones (A-C, 9 div shown) or striatal neurones (D-F, 13 div shown) at each div. For each insult and time-point NAAG (1-30 μM) was not significant from that of insult (One-way ANOVA, $P > 0.05$, $n = 6$-8).

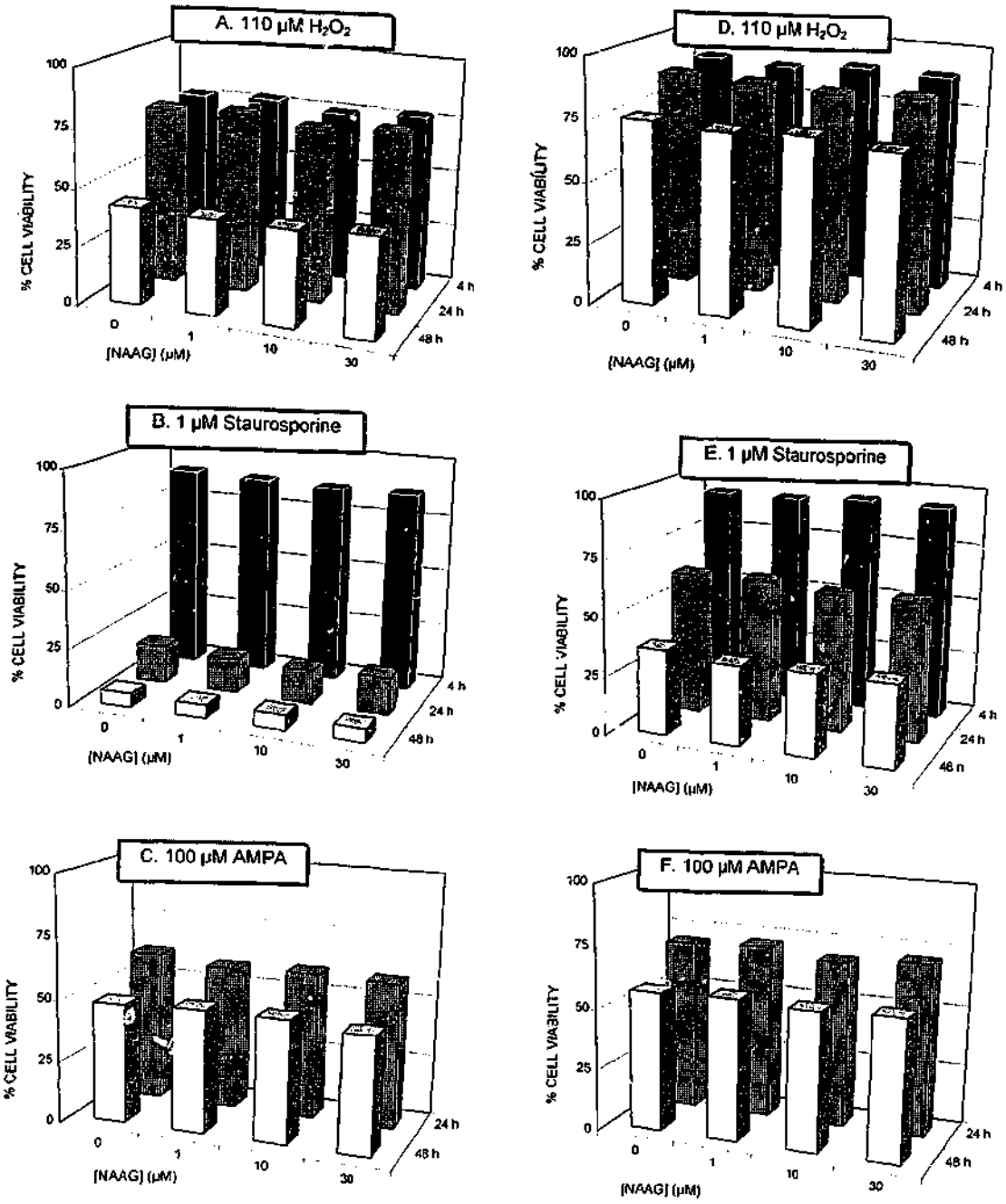

A. 110 µM H_2O_2
D. 110 µM H_2O_2
B. 1 µM Staurosporine
E. 1 µM Staurosporine
C. 100 µM AMPA
F. 100 µM AMPA
% CELL VIABILITY
[NAAG] (µM)
0
1
10
30
4 h
24 h
48 h
46 h
100
75
50
25
0

Table 2.2. The decrease in cell viability produced by low K^+ (5.4 mM KCl) in CGCs at 9 div was not attenuated by 2*R*,4*R*-APDC.

		2*R*,4*R*-APDC (μM)		
Time (h)	Vehicle	1	10	30
4	79.4 ± 1.0	81.1 ± 1.1	80.3 ± 0.3	80.4 ± 0.4
24	31.7 ± 1.5	32.7 ± 0.6	30.7 ± 0.9	33.0 ± 1.0
48	17.9 ± 6.8	15.3 ± 6.1	15.3 ± 7.3	19.8 ± 8.3

Values represent the mean ± S.E.M. (n = 5-6). $P \geq 0.4906$.

Table 2.3. Varying modes of treatment with 2*R*,4*R*-APDC fail to attenuate the decrease in cellular viability induced by various insults in cortical, striatal and cerebellar granular neuronal cultures.

TREATMENT	CELL VIABILITY (% MEDIUM ONLY)					
A. 2 h Preincubation	H_2O_2 (100-110 μM)	Staurosporine (1 μM)	NMDA (70 μM)	KA (100 μM)	AMPA (100 μM)	Low K^+ (5.4 mM KCl)
Cortical:						
Insult	31.5 ± 4.6	22.2 ± 2.8	66.2 ± 3.2	48.3 ± 2.1	57.7 ± 2.6	–
APDC	29.4 ± 2.5	23.2 ± 2.2	69.7 ± 7.3	47.8 ± 2.1	56.0 ± 3.7	
Striatal:						
Insult	86.2 ± 3.6	58.5 ± 1.8	76.8 ± 7.1	71.2 ± 3.1	74.3 ± 3.2	–
APDC	86.7 ± 4.1	59.3 ± 2.1	77.5 ± 6.8	71.6 ± 2.8	74.5 ± 4.8	
Cerebellar:						
Insult	–	–	–	–	–	26.3 ± 0.6
APDC						28.7 ± 1.7
B. 100 μM APDC						
Cortical:						
Insult	19.8 ± 1.8	28.3 ± 3.5	43.6 ± 3.4	48.7 ± 2.6	55.8 ± [illegible]	–
APDC	19.8 ± 0.9	25.4 ± 1.8	46.0 ± 5.2	52.4 ± 0.7	54.5 ± [illegible]	
Striatal:						
Insult	78.3 ± 3.8	61.5 ± 2.4	86.0 ± 2.8	71.9 ± 1.9	62.2 ± 1.3	–
APDC	78.7 ± 1.6	61.7 ± 3.0	84.2 ± 2.9	72.6 ± 2.5	60.6 ± 2.9	
Cerebellar:						
Insult	–	–	–	–	–	30.7 ± 1.7
APDC						30.3 ± 0.9
C. Acute Insult (30 min)	H_2O_2 (150 μM)	Staurosporine (10 μM)	NMDA (300 μM)	KA (1000 μM)	AMPA (1000 μM)	–
Cortical:						
Insult	65.9 ± 3.3	25.8 ± 5.7	55.2 ± 3.2	40.5 ± 1.8	56.1 ± 5.2	–
APDC	69.3 ± 2.2	24.3 ± 3.0	58.4 ± 1.9	39.3 ± 2.2	58.9 ± 2.4	
Striatal:						
Insult	79.2 ± 3.2	55.7 ± 1.6	74.7 ± 4.5	75.9 ± 3.1	77.9 ± 2.3	–
APDC	74.3 ± 2.3	55.3 ± 4.1	74.4 ± 3.0	74.1 ± 0.6	70.2 ± 3.7	

With the exception of B, 2*R*,4*R*-APDC = 30 μM. Values represent the mean ± S.E.M. of 6-8 treatments from 2 independent cultures. Experiments shown are of cortical and cerebellar granular neurones at 9 div and striatal neurones at 13 div following treatment for 24 h. $P \geq 0.066$. (–: not determined).

Figure 2.6 Phase-contrast microscopy showing the loss of morphological integrity from exposure to insults is not prevented by $mGlu_{2/3}$ agonists.

Photomicrographs are of cortical neurones (A-L) or cerebellar granule cells (M-O) at 9 div following treatment for 24 h with 2*R*,4*R*-APDC (30 μM) and/or insult.

A. N2-supplemented medium only.

B. 1 μM staurosporine

C. 1 μM staurosporine in the presence of 2*R*,4*R*-APDC.

D. 100 μM H_2O_2.

E. 100 μM H_2O_2 in the presence of 2*R*,4*R*-APDC.

F. 100 μM KA.

G. 100 μM KA in the presence of 2*R*,4*R*-APDC.

H. 100 μM AMPA

I. 100 μM AMPA in the presence 2*R*,4*R*-APDC.

J. DMEM medium only;

K. 70 μM NMDA

L. 70 μM NMDA in the presence of 2*R*,4*R*-APDC.

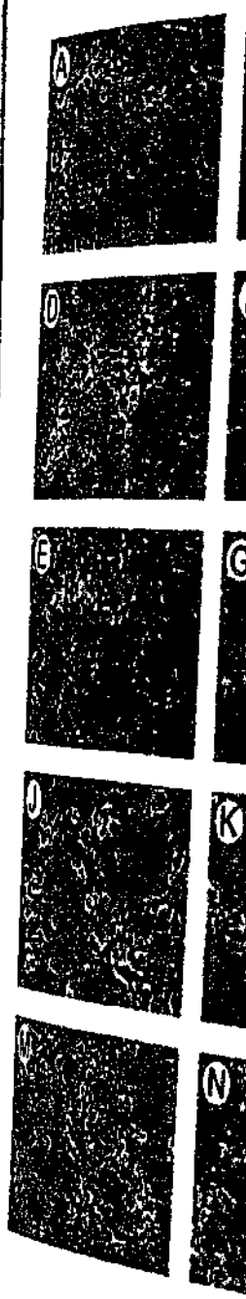
CGCs:

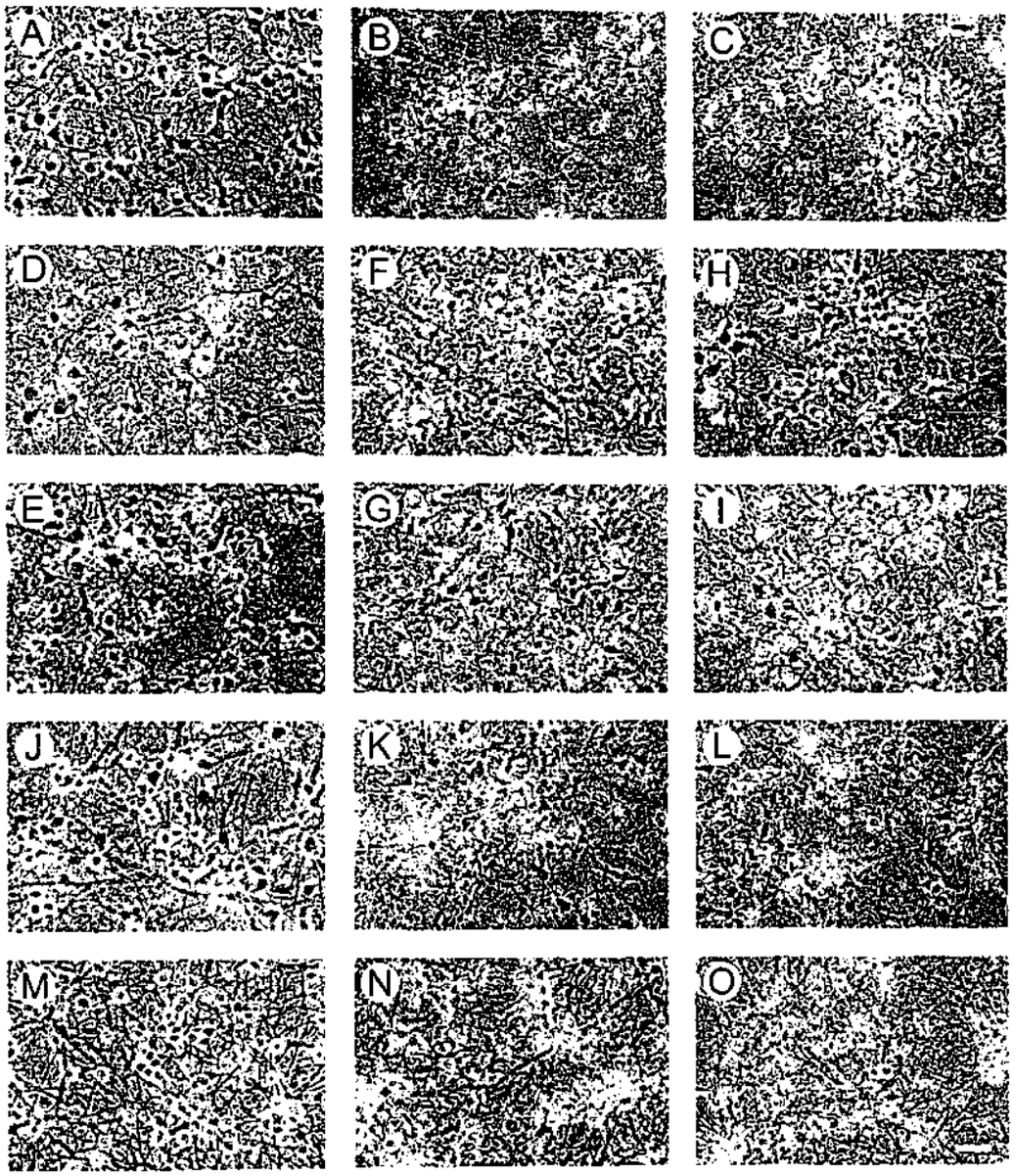
M. Medium only.

N. 5.4 mM KCl.

O. 5.4 mM KCl in the presence of 2*R*,4*R*-APDC.

Scale bar = 75 μm.

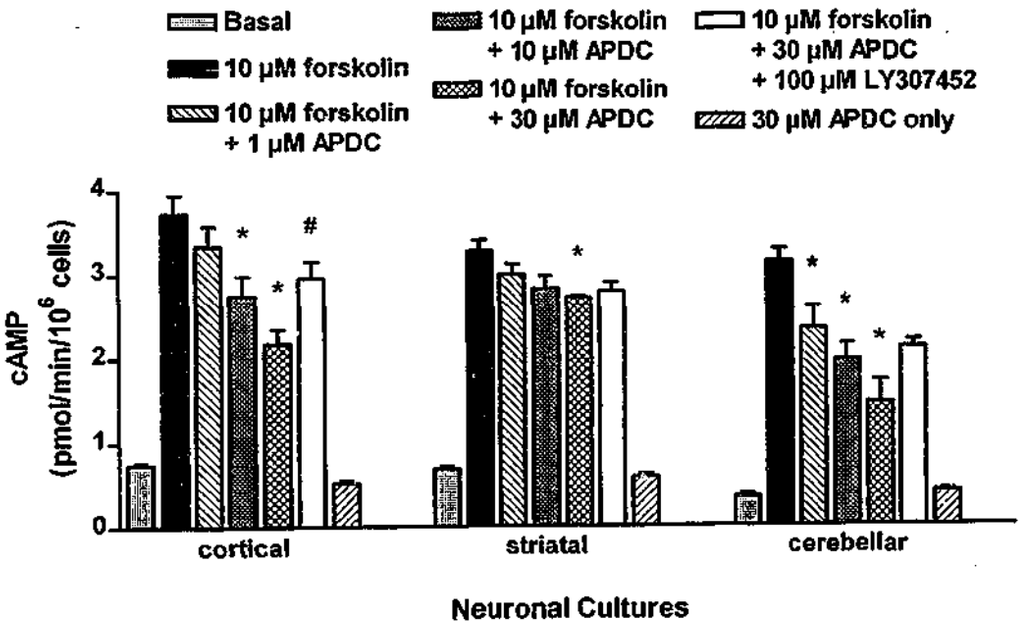



A
B
C
D
F
H
E
G
I
J
K
L
M
N
O

2.3.4. *Inhibition of cAMP formation*

In cortical neuronal cultures, the increase in cAMP production following forskolin stimulation was decreased significantly in a concentration-dependent manner 2*R*,4*R*-APDC ($F_{3, 29} = 8.99$, $P = 0.0003$). The $mGlu_{2/3}$ antagonist LY307452 (Schoepp et al., 1999; 100 μM) significantly attenuated the decrease in cAMP produced by 30 μM 2*R*,4*R*-APDC ($P = 0.0177$; Figure 2.7). In striatal neurones, 2*R*,4*R*-APDC only produced a significant decrease in cAMP formation at 30 μM ($F_{3, 25} = 3.82$, $P = 0.0364$). LY307452 failed to prevent the reduction in cAMP formation by 30 μM 2*R*,4*R*-APDC in these cultures ($P = 0.5173$; Figure 2.7). In CGC neurones 2*R*,4*R*-APDC caused a significant concentration-dependent inhibition of forskolin-stimulated cAMP production ($F_{3, 27} = 9.16$, $P = 0.0003$). While LY307452 caused an approximately 35% reversal of the specific effect of 30 μM 2*R*,4*R*-APDC, this increase was not statistically significant because of the variability of the agonist alone data ($P = 0.0816$; Figure 2.7). In summary, 2*R*,4*R*-APDC (30 μM) decreased the forskolin-stimulated production of cAMP in cortical, striatal and CGC neurones by approximately 41%, 17% and 46% respectively. In the absence of forskolin stimulation, production of cAMP by cultures treated with 30 μM 2*R*,4*R*-APDC did not differ significantly from untreated cultures ($P = 0.568$; Figure 2.7).

Figure 2.7 Concentration-dependent reduction of forskolin-stimulated production of cAMP by 2*R*,4*R*-APDC in pure neuronal cultures at 9-12 div. cAMP values represent the mean ± S.E.M. from duplicate determinations of 3-4 independent experiments. **P* < 0.05 when compared to 10 μM forskolin alone, #*P* < 0.05 when compared to 10 μM forskolin + 30 μM 2*R*,4*R*-APDC.



2.4. Discussion

The key finding of our strategic study was that stimulation of $mGlu_{2/3}$ failed to protect cultured neurones from a range of toxic insults. This conclusion was reached on the basis of a diverse range of investigations employing three different neuronal cultures, various neurotoxic insults and treatment conditions, and despite evidence for the presence of functional $mGlu_{2/3}$. Since the prime aim of the present study was to conduct this investigation into the putative neuroprotective role of $mGlu_{2/3}$ agonists in the virtual absence of glia, and to attempt to resolve some ambiguity in this area, successful cultures were established where glia represented 2-7% of all cells. Under these defined conditions, unlike a number of previous studies, $mGlu_{2/3}$ agonists were never neuroprotective in any of the experimental paradigms investigated.

Positive *in situ* mRNA and protein expression of $mGlu_{2/3}$ has been located in a number of cortical regions, striatum and in the granule cell layer of the cerebellum (Ohishi et al., 1993a,b; Ohishi et al., 1994). Cartmell et al. (1998) detected $mGlu_3$ transcripts by *in situ* hybridisation in rat striatal neurones and showed inhibition of Ca^{2+} currents by DCG-IV in these cultures. Secondly, although low $mGlu_{2/3}$ expression was found by Prezeau et al. (1994) in rat striatal neurones, marked protein expression was found in rat cortical and cerebellar granular neuronal cultures. Additionally, despite evidence indicating predominant $mGlu_{2/3}$ expression in glia, Makoff et al., (1996a) showed that the expression of human $mGlu_3$ throughout the cerebellum and cerebral cortex predominated in neurones. Finally, NAAG-like immunoreactivity (Passani et al., 1997) and ^{3}[H]NAAG binding (Shave et al., 2000) has been shown in cortical, striatal and cerebellar granular regions also. In the findings not presented here, positive $mGlu_3$-like immunoreactivity was found to be neuronally localised within each culture at each div using a previously characterised

antibody directed at $mGlu_3$. This antibody has demonstrated widespread neuronal localisation of $mGlu_3$ -like immunoreactivity in brain, including the striatum (Beart et al., 1999) in agreement with both previous *in vivo* and *in vitro* studies.

Evaluation of the neuroprotective potential of $mGlu_{2/3}$ agonists required investigating a variety of toxic insults. Injury to cortical and striatal neurones induced by NMDA, AMPA and KA was generally not substantial after 4 h exposure, so cultures subjected to these insults were examined after 24 and 48 h. Staurosporine, H_2O_2 and low K^+ did induce substantial neurotoxicity after 4 h and hence cultures used for the evaluation of the actions of $mGlu_{2/3}$ agonists included this timepoint in addition to 24 and 48 h. The degree to which insults decreased cellular viability greatly depended on the type of culture and the div on which the experiments were carried out. For example, striatal neurones at 6 div were more vulnerable to H_2O_2 insult than cortical neurones at the same div, consequently 60 μM H_2O_2 was used for striatal neurones at 6 div, but 70 μM for cortical neurones. These differential actions of H_2O_2 were most likely due to the comparatively slow development of the neuronal monolayer observed in striatal neurones at this timepoint. In contrast, the reverse applied at 13 div, where 110 μM H_2O_2 was used for striatal neurones but 100 μM for cortical neurones. In this case the increased background cell death observed in cortical neurones most likely contributed to an increased sensitivity to H_2O_2 insult. Regardless of the div, striatal neurones were less susceptible to excitatory amino acid toxicity than cortical neurones. This observation was not unexpected considering inhibitory GABAergic neurones predominate in the striatum, where the only glutamatergic neurones are extrinsic and associated with thalamostriatal and corticostriatal projections.

In general, DCG-IV, L-CCG-I and 2*R*,4*R*-APDC possess similar potencies at $mGlu_{2/3}$ (Schoepp et al., 1999). However, DCG-IV and L-CCG-I also exhibit agonist

activity at NMDA receptors and group I mGlu receptors respectively. Hence, 2*R*,4*R*-APDC is the most potent and selective $mGlu_{2/3}$ agonist used in the present study. Considering the cultures employed express native mGlu receptors this feature is of particular importance. NAAG is less potent than 2*R*,4*R*-APDC, but possesses particular selectivity for $mGlu_3$ versus $mGlu_2$ receptors (Schoepp et al., 1999).

In mixed cortical cultures DCG-IV has been shown to attenuate both NMDA- and KA-induced toxicity across a concentration range of 10 nM to 100 μM (Bruno et al., 1995a). Similarly, L-CCG-I, LY354740 and its analogues have been shown to attenuate NMDA injury but from concentrations >1 μM (Bruno et al., 1994; Kingston et al., 1999; Behrens et al., 1999). Employing these effective concentrations in cortical pure neuronal cultures, DCG-IV and L-CCG-I (in the presence of appropriate antagonists) failed to protect neurones against both acute and chronic toxicity induced by NMDA, AMPA and KA. In agreement with the present study DCG-IV did not protect against chronic NMDA-induced toxicity in mixed cultures (Buisson et al., 1996). Similarly, 2*R*,4*R*-APDC (> 0.1 μM) has been shown to be neuroprotective in mixed cultures exposed to NMDA insult (Battaglia et al., 1998; Kozikowski et al., 1999), although when used at these concentrations in the present study neuroprotection was not observed throughout the various experimental strategies. NAAG, also failed to protect neurones from toxic insult despite earlier reports to the contrary in mixed cultures (Bruno et al., 1998b). Furthermore, the agonist concentrations were increased to 100 μM and 300 μM (30,000 times the minimal effective concentration and equivalent to the maximal effective concentration used in mixed cultures) without evidence of neuroprotection. The significance of mixed cultures will be discussed below.

In a recent report, Colwell and Levine (1999) proposed that $mGlu_{2/3}$ modulation of NMDA-induced toxicity involved voltage-operated Ca^{2+} channels (VOCC). Their study revealed that inhibition of Ca^{2+}-influx through VOCC, particularly high-voltage gated N-type Ca^{2+} channels, reduced the degree of neuronal swelling induced by NMDA, and that this effect was mimicked by the non-selective agonist *trans*-1-amino-cyclopentyl-1*S*,3*R*-dicarboxylate (*t*-ACPD). Since cortical, striatal and cerebellar granular regions express N-type Ca^{2+} channels (Chung et al., 2000) it might be expected that the agonists employed here would attenuate NMDA injury. However, our results suggest that either inhibition of the N-type Ca^{2+} channel is incapable of attenuating toxicity alone under the conditions investigated or that the non-selective activity of *t*-ACPD for group I mGlu receptors may contribute to the result seen by Colwell and Levine (1999).

Group II mGlu receptor-mediated neuroprotection has been observed following application of the agonists prior to KA insult in spinal cord slices (Pizzi et al., 2000), mixed cultures (Bruno et al., 1995a) and cortical neuronal cells (Kingston et al., 1999). In the present study, neuroprotection was not observed in cortical or striatal neuronal cultures despite attempts to match both the mode of KA insult and the concentration of $mGlu_{2/3}$ agonists employed by Kingston et al. (1999). This latter study also investigated AMPA-induced toxicity in the presence of cyclothiazide and did not find any neuroprotective effect by the agonists. Our study also failed to demonstrate $mGlu_{2/3}$ agonist neuroprotection against AMPA alone-induced toxicity in cortical and striatal neuronal cultures, observations which also support those findings of Bruno et al. (1995a) in mixed cultures.

Staurosporine is a non-selective protein kinase inhibitor which is often used to induce apoptosis in cultures. Novel $mGlu_{2/3}$ agonists were recently shown to protect against staurosporine-induced toxicity in cortical mixed cultures (Kingston et al., 1999). Once again,

despite our attempts to match the mode of staurosporine toxicity and $mGlu_{2/3}$ agonist concentrations employed by these investigators, neuroprotection was not found in either cortical or striatal neuronal cultures. But whilst 2*R*,4*R*-APDC is not quite as selective as the agonists used by Kingston et al., (1999) it is however equally as potent at group II mGlu receptors as other agonists with reported neuroprotective properties such as DCG-IV, L-CCG-I and NAAG and so is not likely to have affected the outcome of our extensive investigations.

Cellular injury from exposure to H_2O_2 results from a number of effects that are different from EAA- or staurosporine-induced injury, including modulation of Glu exocytosis and oxidative stress (Pellegrini-Giamprieto et al., 1988; Abe and Saito, 1998). Since H_2O_2 is often detoxified by cultured astrocytes (Desagher et al., 1996) the pure neuronal cultures employed in this study provided a sensitive and controlled model for investigating $mGlu_{2/3}$ agonist modulation of oxidative stress. However, none of the strategies investigated revealed neuroprotection by the agonists DCG-IV, L-CCG-I, NAAG or 2*R*,4*R*-APDC. No previous studies have been undertaken investigating $mGlu_{2/3}$-meditated neuroprotection of H_2O_2-induced insult in mixed cultures, however, given the importance glutathione plays in neuronal-glial interactions (Drukarch et al., 1997), $mGlu_{2/3}$ stimulation may be neuroprotective via stimulation of this interaction and/or neurotrophin release following H_2O_2-induced injury in neurones.

Cultured CGCs maintained in a growth medium containing 25 mM KCl undergo apoptosis when changed to a medium containing approximately 5 mM KCl (low K^+). CGCs undergo apoptosis within 2-5 h from transfer into low K^+ involving activation of *c-jun* mRNA expression, caspase-like proteases and DNA fragmentation (review: Ikeuchi et al., 1998). Low K^+-induced apoptosis was induced for 4, 24 and 48 h in this study and the

neuroprotective potential of $mGlu_{2/3}$ agonists investigated. Despite employing a range of concentrations and including pre-incubation of the agonists, neuroprotection was not observed using the cellular viability assay or phase-contrast microscopy. Furthermore, this absence of neuroprotection is not dependent on the development of the cerebellar neurones, since at 6, 9 and 13 div cultures produced similar results. These findings do not disagree with previous evidence which indicates that production, rather than inhibition of cAMP, is responsible for the attenuation of low K^+-induced apoptosis (D'Mello et al., 1993).

Previous studies have demonstrated that following exposure of group II mGlu receptor agonists, 2 h is required before substantial neuroprotection is achieved in mixed cultures undergoing NMDA insult (Bruno et al., 1997). In line with these findings the neuronal cultures in the present study were treated for 2 h with 2*R*,4*R*-APDC prior to the addition of either H_2O_2, staurosporine, NMDA, AMPA or KA for 4, 24 or 48 h. Following this prestimulation, 2*R*,4*R*-APDC was also incubated in the presence of the insults and failed to protect against the injury induced in the cultures.

NMDA-induced toxicity has been suggested to be partly due to an increase in cAMP (Buisson and Choi, 1995; Schaffhauser et al., 1997). In the light of observations that protein kinase A (PKA) activators and dopamine receptors positively coupled to AC enhance NMDA responses (Colwell and Levine, 1996; Cepeda et al., 1998), it might be expected that inhibitors of the AC/PKA pathway, such as $mGlu_{2/3}$ agonists, would attenuate NMDA receptor-mediated toxicity. However, the present study has demonstrated that despite inhibiting cAMP production (via Ca^{2+}-sensitive AC), group II mGlu receptor agonists fail to attenuate not only varying modes of NMDA-induced toxicity but also KA and AMPA-induced toxicity, including pre-inhibition of the AC/PKA pathway.

The importance of glia in mediating neuroprotection has become evident recently. Neuroprotection observed in mixed cultures was identified as being dependent on protein synthesis (Bruno et al., 1997). Subsequent studies showed that neurotrophic factors were produced following $mGlu_3$ stimulation, of which transforming growth factor (TGF)-β1 and -β2 were identified as primary candidates responsible for the glial-mediated neuroprotection observed (Bruno et al., 1998a) ($mGlu_3$-stimulated production of NGF and S100-β has since been demonstrated also; Ciccarelli et al., 1999). More recent studies showed that greater neuroprotection was obtained in mixed cultures than those containing only 15% glia (Kingston et al., 1999), supporting the hypothesis that glial production of trophic factors is responsible for neuroprotection. However, evidence has been emerging that glia are not essential for trophic neuroprotection (Dobbertin et al., 1997; Hattori et al., 1999; Zhu et al., 2000). In particular, TGFβ-1 immunoreactivity has been shown to be upregulated days before glial activation in transient forebrain ischaemia and this immunoreactivity is not co-localised with cells considered to be apoptotic on the basis of TUNEL-positive staining (Zhu et al., 2000). Despite this evidence indicating neuronal production of trophic factors and subsequent neuroprotection, the toxicity induced here was not attenuated in pure neuronal cultures.

The suitability of 2*R*,4*R*-APDC as a selective activator of $mGlu_{2/3}$-mediated intracellular events is clouded by a number of reports which suggest that at high doses 2*R*,4*R*-APDC-induced $mGlu_{2/3}$ activation augments the intracellular response of $mGlu_{1/5}$ activation. This phenomena was originally reported by Schoepp et al., (1996) and has since been reported to be responsible for a number of biphasic responses observed with 2*R*,4*R*-APDC including proconvulsant activity (Attwell et al., 1998a) and neurotoxicity during NMDA insult (Battaglia et al., 1998). In the latter study 2*R*,4*R*-APDC provided less

neuroprotection at 30-100 μM when compared to 1 μM during NMDA insult in mixed cultures. In the present study, 2*R*,4*R*-APDC did not augment the toxicity induced by NMDA nor any other insult investigated. Alternatively, results from the cAMP assay suggest that 2*R*,4*R*-APDC provides concentration-dependent inhibition of cAMP production, including concentrations greater than 1 μM. In fact, significant inhibition of cAMP production by 2*R*,4*R*-APDC was not observed at 1 μM in any of the cultures investigated. Instead there appears to be a correlation between the augmentation of $mGlu_{1/5}$ second messenger systems by 2*R*,4*R*-APDC thought to be observed by various groups and the inhibition of cAMP production by 2*R*,4*R*-APDC demonstrated here. Additionally, the absence of neuroprotection by 2*R*,4*R*-APDC in the present study does not support the hypothesis that activation of pre- and postsynaptic neuronal $mGlu_{2/3}$ results in neuroprotection, rather these results suggest that the neuroprotection provided by $mGlu_{2/3}$ in mixed cultures originates from glia (which supports previous findings: Bruno et al., 1997; Bruno et al., 1998a; Kingston et al., 1999). But more importantly, glia have been shown not to accommodate $mGlu_{2/3}$-mediated inhibition of cAMP production (Prezeau et al., 1994; Bruno et al., 1995a), and yet 1 μM 2*R*,4*R*-APDC was the maximally effective concentration against NMDA-induced toxicity in mixed cultures (Battaglia et al., 1998). Consequently, it is most likely that the neuroprotection observed throughout mixed cultures is not primarily instigated by inhibition of the neuronal or glial AC/cAMP pathway and therefore does not primarily depend on the $mGlu_{2/3}$-mediated regulation of neurotransmitter release. Group II mGlu receptor mediated neuroprotection most likely results from an entirely glial mechanism (especially in light of Bruno et al., 1997, 1998a) which at present remains uncharacterised.

Regardless of the mechanisms purported to be active in the cultures employed throughout the investigation, including Ca^{2+} channel-mediated inhibition of EAA toxicity and cAMP-mediated inhibition of NMDA toxicity, and despite demonstrating the presence of functional $mGlu_{2/3}$, neuroprotection was not observed in the present study. These results add support to the growing body of evidence that $mGlu_{2/3}$ agonist-stimulated glial production of trophic factors is the primary mechanism of neuroprotection observed in studies employing $mGlu_{2/3}$. Finally, this investigation highlights the importance of defining the morphology of models employed for neuroprotection studies, since even subtle morphological differences can influence the outcome observed.

NOTE ADDED:

Some months after these findings were published, D'Onofrio et al. (2001) published their study of the pathways involved in the release of the neuroprotective trophic factor TGF-β as observed earlier by Bruno et al., (1998). In brief, D'Onofrio et al., demonstrated an increase in astrocyte cultures an increase in the phosphorylated forms of extracellular signal-regulated kinase (ERK1/2) and Akt thereby implicating the recruitment of the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3-K) pathways following $mGlu_3$ receptor activation by 4C3HPG and LY379268. Similar agonists were used *in vivo* whereby an increase in TGF-β mRNA was observed, and which could be decreased by inhibition the MAPK pathway. This study was able to link the $mGlu_3$-induced and astrocytic TGF-β-mediated neuroprotective effects that had been noticed *in vivo* to the

neuroprotection of striatal neurones *in vivo*, which Bond et al. (2000) could not demonstrate in a rat model of ischaemia.

The elegant work by D'Onofrio et al. emphasised the importance of group II mGlu receptor function in astrocytes as a key event contributing to neuroprotection in models of neuronal injury. Therefore, it appeared that addressing $mGlu_{2/3}$ function in astrocytes was more relevant to the aims of this thesis than addressing $mGlu_{2/3}$ function in neurones. Consequently, the next series of experiments aimed at investigating the role of cAMP in astrocytes and its role in neuroprotection, particularly since cAMP is the key signal transduction component associated with group II mGlu receptors.

CHAPTER 3

$mGLU_{2/3}$-MEDIATED REGULATION OF cAMP IN NEURONES AND ASTROCYTES

3.1. Introduction

While many groups have demonstrated that $mGlu_{2/3}$ mediate inhibition of forskolin-stimulated production of cAMP, a disparity exists surrounding the conditions in which this inhibitory effect can be achieved. Early research showed that the prototypic mGlu receptor agonists (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD), 1*S*,3*S*-ACPD and 1*S*,3*R*-ACPD decreased forskolin-stimulated production of cAMP in striatal neurones and cerebral slices (Schoepp et al., 1992b; Prezeau et al., 1992; Manzoni et al., 1992; Cartmell et al., 1992). At the same time it was discovered that these agonists could induce increases in cAMP accumulation (Casabona et al., 1992; Schoepp et al., 1992b; Winder and Conn, 1992). It was clear that this effect was not mediated by direct coupling of $mGlu_{2/3}$ to G_s, but rather through indirect potentiation of other G_s-mediated agonist responses. However, the non-selective activity of these early agonists at $mGlu_{1/5}$ was considered likely to indirectly contribute to the activity observed. Nevertheless, potentiation of cAMP production was again observed with the more selective $mGlu_{2/3}$ Carboxycyclopropylglycines DCG-IV and L-CCG-I (Winder and Conn, 1995). In that study, the potentiation of cAMP production was proposed to be mediated by a receptor with $mGlu_{2/3}$-like pharmacology and not via potentiating phosphoinositide hydrolysis as earlier thought (Casabona et al., 1992; Cartmell et al., 1993b; Cartmell et al., 1994). In contrast, Schoepp et al. (1996a) showed that when the $mGlu_{1/5}$ agonist DHPG and the $mGlu_{2/3}$ agonist 2*R*,4*R*-APDC were added in combination to 7-day old rat hippocampal slices a 5-fold potentiation of cAMP production was observed – an effect inhibited by adenosine deaminase and an adenosine receptor antagonist. DCG-IV was without effect in modulating cAMP in slices of rat striatum but inhibited forskolin-stimulated cAMP production in cortical slices (Cartmell et al., 1998; Kemp et al., 1996). Such differences may be explained by varying receptor expression, nevertheless Prezeau et al. (1994) showed mGlu receptor mediated

inhibition of cAMP production in a variety of neuronal and astrocyte cultures, including L-CCG-I-induced inhibition of cAMP production in striatal neurones. In mixed, neuronal-glial cultures Bruno et al. (1994) found no inhibition of cAMP production following application of DCG-IV, L-CCG-I or 1*S*,3*R*-ACPD. Activation of $mGlu_{2/3}$ has been shown to positively modulate phosphoinositide hydrolysis (Genazzani et al., 1994; Schoepp et al., 1996b), while $mGlu_{2/3}$ mediated activity in astrocytes appears to be sensitive to the activation of other G-protein coupled receptors such as adrenoceptors (Balázs et al., 1998a) and adenosine receptors (Di Iorio et al., 1996; Ciccarelli et al., 1999; Cormier et al., 2001).

$mGlu_{2/3}$ have attracted considerable attention as therapeutic targets since they reduce Glu release and inhibit Ca^{2+} entry (review: Cartmell and Schoepp, 2000), two mechanisms likely to contribute to neuronal injury processes including excitotoxicity (Lipton and Rosenberg, 1994) and convulsions in epilepsy (Chapter 4). Agonists of $mGlu_{2/3}$ have been shown to be neuroprotective in animal models of stroke (Bond et al., 1999), *in vivo* models of traumatic brain injury (Allen et al., 1999) and excitotoxicity (Kingston et al., 1999). However, increasing evidence suggests that this neuroprotective mechanism certainly involves $mGlu_{2/3}$ located on glial cells (Bruno et al., 1998; D'Onofrio et al., 2001; Chapter 2). Given that many early investigations of $mGlu_{2/3}$-mediated inhibition of cAMP production were performed in pure neuronal cultures or brain slices where neuronal versus glial events cannot be separated, it is surprising to find that $mGlu_{2/3}$-linked cAMP signalling in astrocytes has received only sparse attention (Balázs et al., 1998a; Cormier et al., 2001).

The present study sought to characterise the *in vitro* pharmacology of the selective $mGlu_{2/3}$ agonists 2*R*,4*R*-APDC and LY379268 by measuring changes in cAMP production in astrocyte and neuronal cultures. Furthermore, our own preliminary evidence (Moldrich and Beart, 2002) had indicated that the ability of $mGlu_{2/3}$ to inhibit stimulated production of cAMP

was sensitive to extracellular calcium. Therefore, further attempts were made to characterise this phenomenon and to elucidate how calcium may affect cAMP responses following activation of $mGlu_{2/3}$.

3.2. Methods

All animal experiments in this chapter were performed according to the Animal Ethics Guidelines of Monash University under animal ethics approval Pharmacology 1999/09. A full list of materials used appears in Appendix IV. Full methods appear in Appendices VI-X.

3.2.1. *Cortical and striatal neuronal cultures*

Cells from the cerebral neocortex and striatum were similarly cultured from Swiss white embryonic mice (day 15-16) as previously described (Cheung et al., 1998b). The cortices and striatum were dissected in Hank's Balanced Salt Solution (HBSS; Appendix III) containing bovine serum albumin (3 mg/ml) and 1.2 mM $MgSO_4$. Tissue was digested with trypsin (0.2 mg/ml) and DNase (80 μg/ml) at 37°C for 5 min. Digestion was terminated by the addition of trypsin inhibitor (0.52 mg/ml) and the cells were triturated and suspended in Neurobasal medium (Appendix I) containing B27 (Appendix II), penicillin (100 U/ml) and streptomycin (100 μg/ml), 0.5 mM glutamine and 10% fetal calf serum. Cells were seeded in Nunc multiwell plates at 0.12×10^6 or 0.3×10^6 cells/well for pharmacological (96-well plates) or morphological/cAMP assay (24-well plates) experiments, respectively. After 24 h *in vitro*, the medium was replaced with serum-free Neurobasal medium containing B27 supplement, and cultures were maintained in a humidified CO_2 incubator (5% CO_2, 8.5% O_2; 37°C) for up to 13 days *in vitro* (div). Partial medium changes were performed each 4-5 div.

3.2.2. *Astrocyte culture*

The forebrains of Swiss white mice (postnatal day 1-2) were dissected, digested and triturated in a manner similar to that of cortical/striatal neuronal cultures. Cells were originally cultured in flasks in 10 ml of Astrocyte Medium (AM) consisting of Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, penicillin/streptomycin (100 U/ml/100 μg/ml) and Fungizone® (amphotericin B, 1 μg/ml) at 36°C and 5% CO_2. Complete medium changes were performed twice weekly until cells were confluent. Following detachment from the flasks with 10 mM EDTA (Ratek Orbital Mixer; 37°C, 200 rpm, 2 h), astrocytes were replated in Nunc multiwell plates at 3.0×10^4 or 8.0×10^4 cells/well for calcium assay (96-well plates) or cAMP assay (24-well plates) experiments. Astrocytes were grown to confluence with twice weekly medium changes at 36°C and 5% CO_2, such that at the time of the experiments astrocytes were 24–28 div. These cultures demonstrate no neuronal microtubule associated protein-2, but >90% glial fibrillary acidic protein-positive, immunoreactivity (representative photomicrographs shown in Appendix IX).

3.2.3. *Measurement of cAMP*

The cellular content of cAMP in cortical neurones, striatal neurones and astrocytes was measured following exposure to 10 μM forskolin in the presence and absence of LY379268. Treatments were prepared in 1.8 mM $CaCl_2$-containing or $CaCl_2$-free HEPES-buffered saline (HBS: 146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% (v/v) glucose, 20 mM HEPES; pH 7.2) containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM). In some experiments the extracellular calcium concentration ($[Ca^{2+}]_e$) was adjusted as indicated or replaced with 1.8 mM $BaCl_2$. Cultures were washed with appropriate buffers prior to 5 min

exposure to treatment solutions at 36/37°C. Vehicle (IBMX) and drug pre-incubation treatments were performed where appropriate.

The treatment solutions were aspirated and the cellular cAMP extracted by scraping the well in the presence of ice-cold 70% ethanol and 1 mM EDTA. This extract was gently centrifuged at 37°C (Savant Environmental Speedvac ESC2000) to evaporate the supernatant and the pellet was resuspended in cold 50 mM Tris-1mM EDTA solution. Aliquots of the sonicated resuspension were assayed according to the commercially available Biotrak [^{3}H]cAMP assay kit (Amersham). The protein content of the final resuspension was determined using the Bio-Rad D_C Protein Assay kit using BSA as standard.

3.2.4. *Intracellular calcium concentration*

The intracellular calcium concentration ($[Ca^{2+}]_i$) of cortical neurones and astrocytes was measured in an attempt to correlate $[Ca^{2+}]_i$ with the cAMP responses observed. The $[Ca^{2+}]_i$ was determined using the Fluo-3/AM calcium-binding dye. Cells were loaded with 10 μM Fluo-3/AM in HEPES-buffered saline containing (mM): NaCl 135, KCl 5, $MgSO_4$ 0.62, $CaCl_2$ 1.8, HEPES 10 and glucose 6, pH 7.4 at 37/36°C for 1 h. DMSO (1%) and 0.2% Pluronic F-127 were included to aid dispersion of the dye. The cells were washed with the above HEPES-buffered saline including 1 mM furosemide to prevent efflux of the dye (used in each buffer thereafter). Cells were washed in either 1.8 mM Ca^{2+}-containing or Ca^{2+}-free HBS (as per section 2.3), followed by a 5 min incubation with IBMX in the presence and absence 10 μM forskolin plus LY379268. The relative fluorescence units (RFU) of the cells was measured after the wash and during treatment at 485/530 nm (excitation/emission) using the Fluoroskan Ascent fluorometer (Labsystems).

3.2.5. *Statistics*

The formation of cAMP was expressed as mean production (pmol/mg protein/min) pooled from 6-9 replicates from 2-3 independent cultures. Basal and 10 μM forskolin treatments were preformed for each experiment and represent 0% and 100% controls respectively, unless otherwise indicated. EC_{50} values were calculated by non-linear regression analysis (sigmoidal, variable slope; GraphPad Prism).

To determine $[Ca^{2+}]_i$, cells were incubated in the presence of the calcium ionophore A23187 (10 μM) following drug treatment to obtain the maximum RFU of the cells (F_{max}) which was quenched with 2 mM $CuCl_2$ to obtain the minimum RFU of the cells (F_{min}). Fluorescence was blanked against unloaded cells, and the free cytosolic calcium concentration determined according to the equation: $[Ca^{2+}]_i = K_D(F\text{-}F_{min})/(F_{max}\text{-}F)$, where F was the observed RFU. K_D was determined to be 145 nM, using a commercially available calibration kit. Data from cAMP and $[Ca^{2+}]_i$ measurements were shown to be from a single population (one-way ANOVA) and significant differences were identified using one-way or two-way ANOVA followed by a Student Newman-Keuls *post-hoc* test, or unpaired *t*-test (SigmaStat) where $P < 0.05$.

3.3. Results

3.3.1. *$mGlu_{2/3}$ pharmacology in neurones*

In cortical neurones 2*R*,4*R*-APDC (Figure 3.1, Table 3.1) and LY379268 (Figure 3.2, Table 3.1) produced a concentration-dependent inhibition of forskolin (10 μM)-stimulated production of cAMP in the absence and presence of 1.8 mM Ca^{2+}; under these conditions EC_{50} values were approximately 0.5 μM and 1.8 μM respectively for 2*R*,4*R*-APDC, and 10 nM and 60 nM, respectively, for LY379268 (Table 3.1). The abilities of 2*R*,4*R*-APDC and

LY379268 to inhibit forskolin stimulated production of cAMP (E_{max} ~12% versus ~23% of control, respectively) were not shown to be significantly different ($P > 0.05$, *t*-test). The presence of Ca^{2+} (1.8 mM) compared with Ca^{2+}-free buffer, failed to significantly influence the efficacy or apparent potency of 2*R*,4*R*-APDC or LY379268 in these cortical neuronal cultures (both agonists: $P > 0.05$, two-way ANOVA).

In striatal neurones, LY379268 (Figure 3.2, Table 3.1) produced a concentration-dependent inhibition of forskolin (10 μM)-stimulated production of cAMP in the absence and presence of Ca^{2+} (1.8 mM) whereby the EC_{50} values were approximately 36 nM and 20 nM, and E_{max} values approximately 65% and 60% of control, respectively (Table 3.1). The presence of Ca^{2+} (1.8 mM) failed to significantly influence the efficacy or potency of LY379268 in these striatal neuronal cultures ($P > 0.05$, two-way ANOVA). The ability of 2*R*,4*R*-APDC to inhibit forskolin-stimulated production of cAMP in striatal neurones has been earlier described in part (Chapter 2).

3.3.2. *$mGlu_{2/3}$ pharmacology in astrocytes*

In astrocytes, 2*R*,4*R*-APDC (Figure 3.1, Table 3.1) and LY379268 (Figure 3.2, Table 3.1) produced a concentration-dependent inhibition of forskolin (10 μM)-stimulated production of cAMP in the absence of extracellular Ca^{2+} to approximately 50% and 30% of control, respectively (Table 3.1). However, unlike cortical or striatal neurones, the presence of Ca^{2+} (1.8 mM) produced a concentration-dependent potentiation of cAMP production in astrocytes by both 2*R*,4*R*-APDC and LY379268 to approximately 200% of control such that there was a significant difference between 1.8 mM Ca^{2+}-containing and Ca^{2+}-free conditions (both agonists: $P < 0.0001$, two-way ANOVA). The apparent potencies of 2*R*,4*R*-APDC and LY379268 to induce this potentiation were approximately 4 and 15 orders of magnitude

Figure 3.1 Concentration-response curves of cAMP production modulated by the $mGlu_{2/3}$ agonist 2R,4R-APDC in murine cortical neurons and forebrain astrocytes.

Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, values adjusted to reflect cAMP production per min and finally normalised against 10 µM forskolin (100% control) in Ca^{2+}-containing (closed circles) or Ca^{2+}-free buffer (open squares) where appropriate.

A. Results show 2R,4R-APDC induced a concentration dependent potentiation of cAMP production in astrocytes in the presence of 1.8 mM Ca^{2+}.

B. Such potentiation is not obtained in cortical neurons. * $P < 0.05$ compared to the same concentration of LY379268 in the absence of 1.8 mM Ca^{2+} (two-way ANOVA, *post hoc* test, n = 6-9).

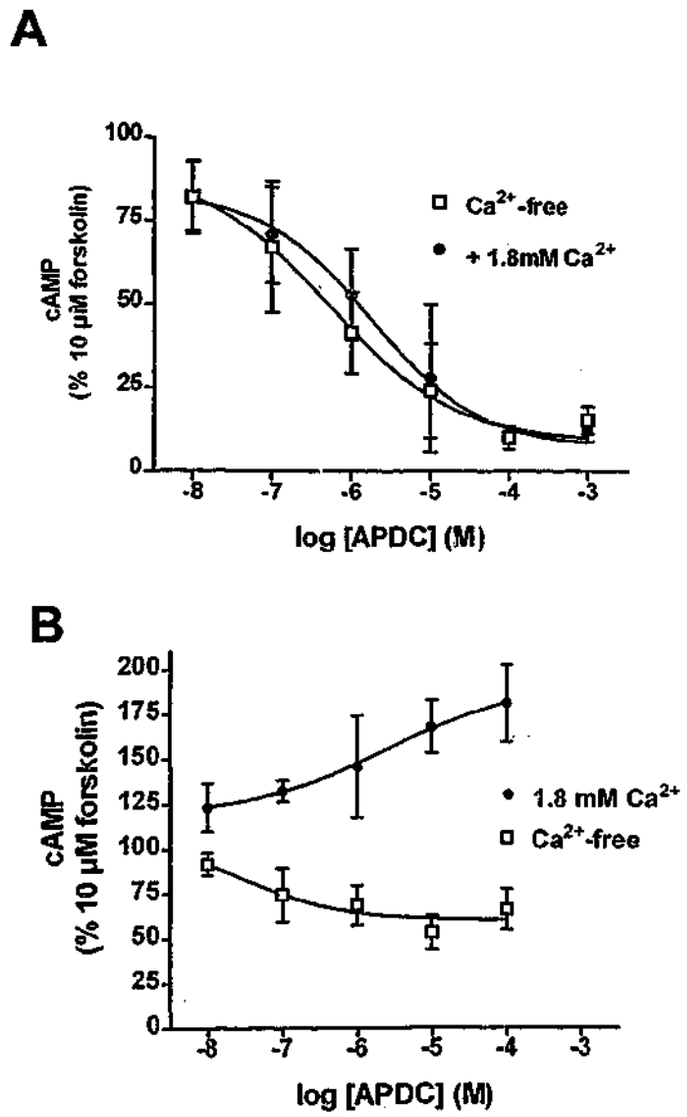
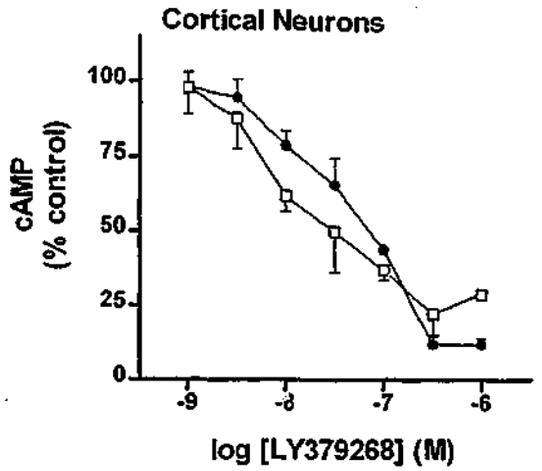
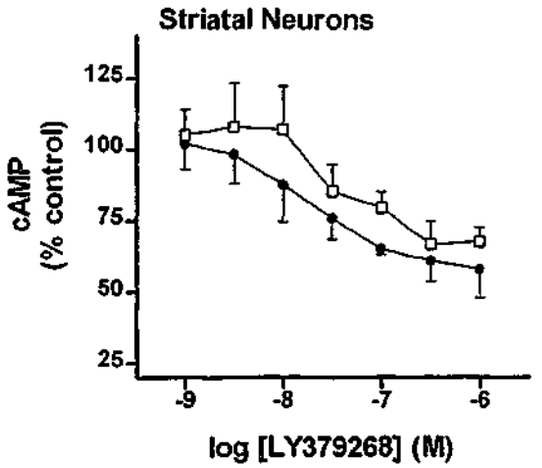

A
100
75
50
25
0
cAMP
(% 10 µM forskolin)
Ca2+-free
+ 1.8mM Ca2+
-8
-7
-6
-5
-4
-3
log [APDC] (M)
B
200
175
150
125
100
75
50
25
0
cAMP
(% 10 µM forskolin)
1.8 mM Ca2+
Ca2+-free
-8
-7
-6
-5
-4
-3
log [APDC] (M)

Figure 3.2 Concentration-response curves of cAMP production modulated by the $mGlu_{2/3}$ agonist LY379268 in murine cortical neurons, striatal neurons and forebrain astrocytes.

Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, values adjusted to reflect cAMP production per min and finally normalised against 10 µM forskolin (100% control) in Ca^{2+}-containing (closed circles) or Ca^{2+}-free buffer (open squares) where appropriate. Results show LY379268 induced a concentration dependent potentiation of cAMP production in astrocytes in the presence of 1.8 mM Ca^{2+}. Such potentiation is not obtained in cortical or striatal neurons. * $P < 0.05$ compared to the same concentration of LY379268 in the absence of 1.8 mM Ca^{2+} (two-way ANOVA, *post hoc* test, n = 6-9).


Cortical Neurons
cAMP (% control)
100
75
50
25
0
-9
-8
-7
-6
log [LY379268] (M)


Striatal Neurons
cAMP (% control)
125
100
75
50
25
-9
-8
-7
-6
log [LY379268] (M)

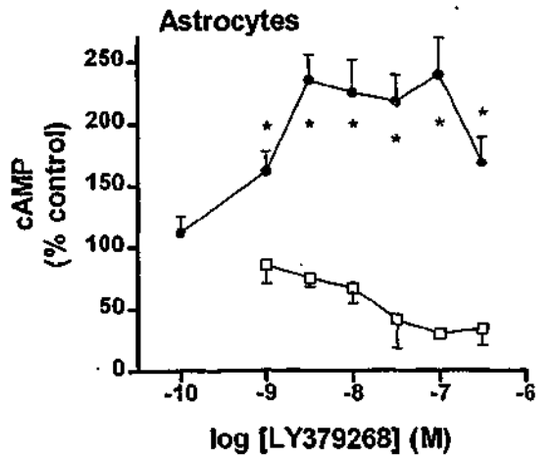

Astrocytes
cAMP (% control)
250
200
150
100
50
0
-10
-9
-8
-7
-6
log [LY379268] (M)

Table 3.1 Modulation of stimulated cAMP in neurones and astrocytes by $mGlu_{2/3}$ agonists.

	$mGlu_{2/3}$ agonist pharmacology in cell culture		
	$[Ca^{2+}]_e$ (mM)	EC_{50} (nM)	E_{max} % of control (μM)
Cortical Neurones			
2R,4R-APDC	0	540 ± 200	8 ± 5 (100)
	1.8	1800 ± 600	7 ± 4 (100)
LY379268	0	10.3 ± 5.4	23 ± 4 (0.3)
	1.8	61.7 ± 20.0	12 ± 2 (0.3)
Striatal Neurones			
LY379268	0	36.2 ± 9.4	65 ± 5 (0.3)
	1.8	19.6 ± 2.9	60 ± 7 (0.3)
Astrocytes			
2R,4R-APDC	0	940 ± 100	50 ± 6 (1)
	1.8	240 ± 100*	194 ± 9 (200)
LY379268	0	15.8 ± 2.5	30 ± 4 (0.1)
	1.8	0.88 ± 0.20*	228 ± 13 (0.1)

$[Ca^{2+}]_e$: extracellular Ca^{2+} concentration. EC_{50} and E_{max} were determined by non-linear regression analysis (sigmoidal, variable slope) using GraphPad Prism. Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, values adjusted to reflect cAMP production per min and finally normalised against 10 μM forskolin (100% control) in Ca^{2+}-containing or Ca^{2+}-free buffer where appropriate. Value in parenthesis represents the concentration (μM) at which a maximal response was observed. * $P < 0.05$ compared to the EC_{50} of either 2*R*,4*R*-APDC or LY379268 in astrocytes in Ca^{2+}-free buffer (*t*-test). n = 6-9.

greater than that required to inhibit forskolin-stimulated cAMP production under Ca^{2+}-free conditions (Table 3.1), which was statistically significant for both agonists ($P \leq 0.0006$, *t*-test).

3.3.3. *The effect of extracellular calcium concentration ($[Ca^{2+}]_e$) on cAMP production and intracellular calcium concentration ($[Ca^{2+}]_i$) in astrocytes*

The dichotomy of $mGlu_{2/3}$ responses in astrocytes was further investigated using the more potent $mGlu_{2/3}$ agonist LY379268 and by manipulating the $[Ca^{2+}]_e$. The inhibition of stimulated cAMP production mediated by $mGlu_{2/3}$ was examined over a range of extracellular Ca^{2+} concentrations (0.001-10 mM). Firstly, forskolin (10 μM)-stimulated production of cAMP was inhibited in Ca^{2+}-free buffer by the inclusion of LY379268 (100 nM; 100% control). Secondly, astrocytes were exposed to increasing $[Ca^{2+}]_e$ which resulted in increases in cAMP production to nearly 500% of control at 3 mM (Figure 3.3A)($P < 0.0001$, one-way ANOVA). The EC_{50} for $[Ca^{2+}]_e$ to potentiate the induced decrease in cAMP production was determined to be 36 ± 10 μM.

A second series of experiments determined that increasing $[Ca^{2+}]_e$ produced increases in $[Ca^{2+}]_i$ in astrocytes based on fluorescence measurements of the affinity of Fluo-3/AM for cytosolic free Ca^{2+} (Figure 3.3B)($P < 0.0001$, one-way ANOVA). While 1 mM and 10 mM $[Ca^{2+}]_e$ produced increases in $[Ca^{2+}]_i$ of approximately 140% and 350% above zero $[Ca^{2+}]_e$, respectively, only the increase in $[Ca^{2+}]_i$ produced by 10 mM was statistically significant ($P < 0.05$, one-way ANOVA, *post hoc* test).

Finally, the combined effect of increasing $[Ca^{2+}]_e$ in the presence of 10 μM forskolin and 100 nM LY379268 was investigated (Figure 3.3C). Increasing $[Ca^{2+}]_e$ produced an increase in $[Ca^{2+}]_i$ whereby both 0.1 mM and 1 mM significantly increased $[Ca^{2+}]_i$ above zero $[Ca^{2+}]_e$ ($P < 0.05$,one-way ANOVA, *post hoc* test). Supramaximal Ca^{2+} (10 mM) decreased $[Ca^{2+}]_i$ beyond

Figure 3.3 Increasing $[Ca^{2+}]_e$ increases cAMP production in astrocytes.

A. Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, values adjusted to reflect cAMP production per min and finally normalised against 10 μM forskolin plus 100 nM LY379268 in the absence of Ca^{2+} (100% control; n = 4). 10 μM forskolin alone (open circle) is in included as reference.

B. Increasing $[Ca^{2+}]_e$ induce $[Ca^{2+}]_i$ increases in astrocytes. $[Ca^{2+}]_i$ measurements represent the mean ± S.E.M. (n = 8) at each $[Ca^{2+}]_e$ over a period of 5 min at 37°C.

* $P < 0.05$ compared to zero $[Ca^{2+}]_e$, one-way ANOVA, *post hoc* test. C. Increasing $[Ca^{2+}]_e$ induce $[Ca^{2+}]_i$ increases and decreases in astrocytes in the presence of 10 μM forskolin and 100 nM LY379268. All $[Ca^{2+}]_i$ are expressed as a ratio of the $[Ca^{2+}]_i$ prior to the addition of 10 μM forskolin plus 100 nM LY379268 to give change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$).

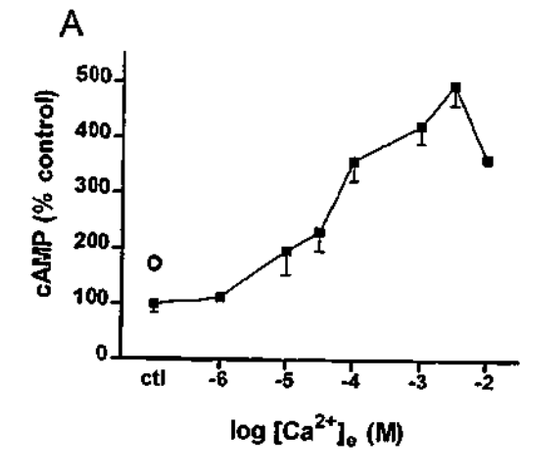
** $P < 0.05$ compared to zero $[Ca^{2+}]_e$, two-way ANOVA, *post hoc* test.

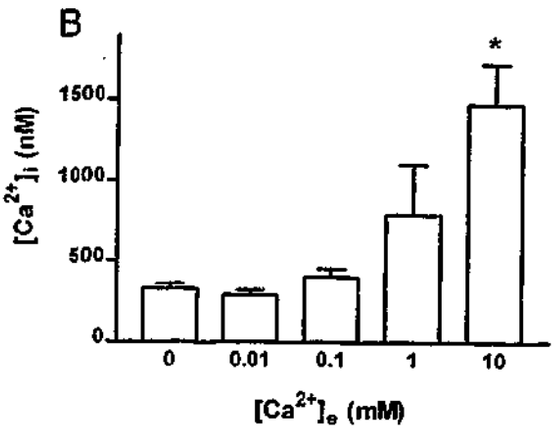
# $P < 0.05$ compared to 1 mM $[Ca^{2+}]_e$, two-way ANOVA, *post hoc* test.

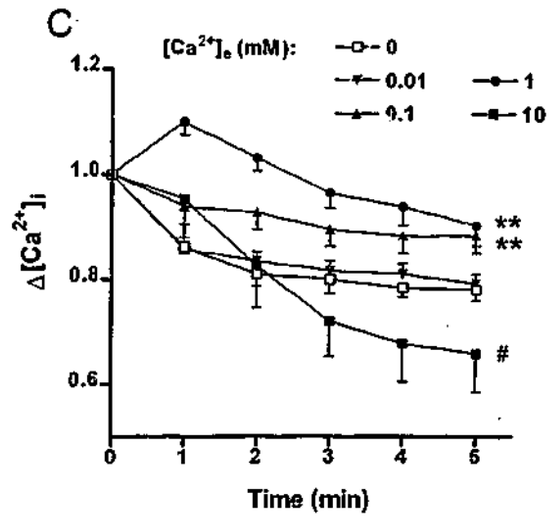
the protein
and finally
Ca^{2+} (100%

easurements

. Increasing
uM forskolin
e addition of







that of 1 mM ($P < 0.05$, one-way ANOVA, *post hoc* test) and slightly beyond that of zero $[Ca^{2+}]_e$ ($P > 0.05$, one-way ANOVA, *post hoc* test), implicating a biphasic and $[Ca^{2+}]_e$-dependent mechanism for modulation of $[Ca^{2+}]_i$ during forskolin and $mGlu_{2/3}$ agonist treatment. This effect correlates with the potentiation, then de-potentiation of forskolin-stimulated production of cAMP by increasing $[Ca^{2+}]_e$ (seen in Figure 3.3A) where 10 mM $[Ca^{2+}]_e$ produced a sub-maximal effect.

3.3.4. *Pharmacological and biochemical modulation of $mGlu_{2/3}$-mediated potentiation or inhibition of forskolin-stimulated cAMP production in astrocytes*

In an attempt to determine the mechanism by which $[Ca^{2+}]_e$ affected the $mGlu_{2/3}$-mediated potentiation or inhibition of forskolin-stimulated cAMP production, a number of receptor agonists and intracellular enzyme inhibitors were employed (Table 3.2). For each treatment, the drug was incubated with astrocytes alone (0% control), in the presence of 10 μM forskolin (100%) and finally, with forskolin and 100 nM LY37928. In the presence of Ca^{2+} (1.8 mM), LY379268 (100 nM) and forskolin (10 μM) produced a potentiation of cAMP production to approximately 240% compared to forskolin alone. In the absence of Ca^{2+}, 100 nM LY379268 and 10 μM forskolin produced an inhibition of cAMP production to approximately 60% compared to forskolin alone (section 3.3.2). Both the potentiation and inhibition of cAMP production was prevented by uncoupling the G_i protein from the $mGlu_{2/3}$ receptor by overnight treatment with 100 ng/ml pertussis toxin to 66% and 130% of respective controls ($P > 0.05$ compared to respective forskolin controls; t-test). Similarly, the potentiation and inhibition was antagonised by the $mGlu_{2/3}$-specific antagonist LY341495 (1 μM; 118% and 85% of respective controls; $P > 0.05$, t-test), and not by the $mGlu_5$-specific antagonist MPEP (1 μM; 247% of control; $P = 0.0163$, t-test). The potentiation of cAMP

production was reduced by the breakdown of endogenous, extracellular adenosine using 1 U/ml adenosine deaminase (134% of control; P = 0.0418, t-test), and reduced by the A_{2A} receptor antagonist ZM241385 (Ongini et al., 1999; 1 μM; 99% of control; P > 0.05, t-test). Furthermore, 30 min pre-incubation of astrocytes with the PLC inhibitor U73122 (10 μM) and the protein kinase A (PKA) inhibitor H89 (30 μM) elevated cAMP formation by forskolin (both inhibitors: P < 0.0002, t-test), however only pre-incubation with U73122 and the calmodulin kinase II inhibitor KN-62 (10 μM) prevented or inhibited $mGlu_{2/3}$-mediated potentiation of cAMP production to 93% (P > 0.05, t-test) and 49% (P = 0.001, t-test) of respective controls, indicating the specific role of these intracellular enzymes in the $mGlu_{2/3}$-mediated potentiation observed.

In 1.8 mM Ca^{2+}-containing buffer, the L-type Ca^{2+} channel blockers nifedipine (1 μM) and nimodipine (1 μM) reduced the LY379268-induced potentiation of cAMP production to 107% of control ($P > 0.05$, *t*-test). In Ca^{2+}-free buffer, the Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) induced $mGlu_{2/3}$-mediated potentiation of forskolin-stimulated production of cAMP to 176% of forskolin alone ($P = 0.0014$, *t*-test). Finally, extracellular Ca^{2+} was replaced by an equal concentration of $BaCl_2$ (1.8 mM) which failed to produce $mGlu_{2/3}$-mediated potentiation of forskolin-stimulated cAMP production (105%; $P > 0.05$, *t*-test), but did reduce the LY379268-induced inhibition of forskolin-stimulated cAMP production. In separate experiments in Ca^{2+}-free buffer (not shown), $BaCl_2$ produced a $[Ca^{2+}]_i$ of 210 ± 17 nM – an increase of approximately 30% above that produced by Ca^{2+}-free buffer. This increase in $[Ca^{2+}]_i$ most likely contributes to the reduction in LY379268-induced inhibition of stimulated cAMP production observed, and possibly results from the ability of $BaCl_2$ to inhibit inward rectifying K^+ currents in astrocytes (Tse et al., 1992). Together, these latter experiments further

Table 3.2 Modulation of $mGlu_{2/3}$-mediated potentiation or inhibition of forskolin (10 μM)-stimulated production of cAMP in astrocytes.

	cAMP Production in Astrocytes (pmol/mg protein/min)		
	Basal	10 μM Forskolin	Forskolin + 100 nM LY379268 (% of control)
+ 1.8 mM Ca^{2+}			
	0.1 ± 0.3	10.1 ± 1.9	24.7 ± 5.2 (240)#
100 ng/ml PTX[a]	0.2 ± 0.2	6.1 ± 0.7	4.1 ± 0.5 (66)
1 μM LY341495	1.1 ± 0.1	12.8 ± 2.3	15.0 ± 3.0 (118)
1 μM MPEP	0.4 ± 0.1	11.3 ± 3.1	27.4 ± 2.7 (247)*
1 U/ml aden. deaminase	0.3 ± 0.1	7.6 ± 1.4	10.1 ± 1.0 (134)*
1 μM ZM241385	0.8 ± 0.3	13.3 ± 3.1	13.2 ± 4.4 (99)
30 μM H89[b]	1.3 ± 0.2	25.4 ± 2.4‡	47.9 ± 11.9 (193)*
10 μM U73122[b]	1.7 ± 0.3	28.0 ± 2.0‡	26.1 ± 7.1 (93)
10μM KN-62[b]	1.2 ± 0.1	13.0 ± 1.0	6.4 ± 0.1 (49)*
Ca^{2+}-channel blockers	0.6 ± 0.2	5.5 ± 0.1	5.9 ± 1.3 (107)
Ca^{2+}-free			
	1.7 ± 0.7	8.3 ± 0.3	5.0 ± 0.4 (58)#
100 ng/ml PTX[a]	0.4 ± 0.1	4.4 ± 0.8	5.6 ± 1.0 (130)
1 μM LY341495	1.2 ± 0.4	9.1 ± 0.7	7.9 ± 1.1 (85)
1 μM thapsigargin	0.9 ± 0.2	5.1 ± 0.3	8.3 ± 0.4 (176)*
1.8 mM $BaCl_2$	0.8 ± 0.3	8.0 ± 0.2	8.4 ± 0.1 (105)

Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, and values adjusted to reflect cAMP production per min. [a] Cells were treated with PTX overnight. [b] H89, U73122 and KN-62 were pre-incubated with astrocytes for 30 min prior to forskolin stimulation. Ca^{2+}-channel blockers include 1 μM nifedipine and 1 μM nimodipine and were included in the wash buffers also. # $P < 0.05$ compared to forskolin, as determined in section 3.2. * $P < 0.05$ compared to forskolin (*t*-test). ‡ $P < 0.05$ compared to forskolin without H89 or U73122 (*t*-test). n = 6-9.

demonstrate the importance of the mobilisation of extracellular and intracellular Ca^{2+} into the cytosol where $mGlu_{2/3}$ second messenger signalling occurs.

3.3.5. *The effect of $[Ca^{2+}]_e$ and pharmacological treatments on $[Ca^{2+}]_i$ in neurones and astrocytes*

Given the importance of both $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$, further studies explored in detail the changes of $[Ca^{2+}]_i$ following treatment of neurones and astrocytes with forskolin, LY379268, and other putative $[Ca^{2+}]_i$ modulators. Since the objective of these experiments was to replicate the conditions used to measure changes in cAMP production, 1 mM IBMX was used throughout treatments. Consequently, the $[Ca^{2+}]_i$ decreased slightly following basal treatment across both neurones and astrocytes by approximately 20% in Ca^{2+}-free buffer, and by approximately 10% in 1.8 mM Ca^{2+}-containing buffer (Figure 3.4). As an additional control, an increase in $[Ca^{2+}]_i$ was induced in both neurones (Figure 3.4A) and astrocytes (Figure 3.4D) with a depolarising concentration of KCl (50 mM), demonstrating the extent and absence of K^+-induced Ca^{2+} influx in Ca^{2+}-containing and Ca^{2+}-free buffer respectively. Treatment of neurones or astrocytes in Ca^{2+}-free buffer with 10 μM forskolin decreased $[Ca^{2+}]_i$ slightly compared to basal (astrocytes: $P < 0.05$, one-way ANOVA, *post hoc* test; Figure 3.4B and E), however treatment with both forskolin and 100 nM LY379268 increased $[Ca^{2+}]_i$ compared to basal (astrocytes: $P < 0.05$, one-way ANOVA, *post hoc* test; Figure 3.4B and E). In contrast, in Ca^{2+}-containing buffer, treatment of neurones with 10 μM forskolin increased $[Ca^{2+}]_i$ slightly compared to basal values ($P < 0.05$, one-way ANOVA, *post hoc* test; Figure 3.4C). This increase in $[Ca^{2+}]_i$ was inhibited in the presence of 100 nM LY379268 ($P < 0.05$, one-way ANOVA, *post hoc* test; Figure 3.4C). There was no significant difference between forskolin and basal, and forskolin plus LY379268 and basal in Ca^{2+}-containing buffer in astrocytes ($P > 0.05$, one-way ANOVA, *post hoc* test; Figure 3.4F).

Since the Ca^{2+}-dependent potentiation of cAMP production was shown to be associated with astrocytes, and not cortical or striatal neurones, further experiments focused on these astrocyte cultures. Consequently, it was demonstrated that the increased $[Ca^{2+}]_i$ in astrocytes in Ca^{2+}-containing buffer was largely mediated by L-type Ca^{2+} channels since the specific L-type Ca^{2+} channel blockers nifedipine (1 μM) and nimodipine (1 μM) inhibited this increase $[Ca^{2+}]_i$ by approximately 75% ($P = 0.0593$, *t*-test; Fig 3.4D).

While the above experiments had established the essential role of $[Ca^{2+}]_e$ in $mGlu_{2/3}$-mediated potentiation of cAMP production in astrocytes, it was unclear if $[Ca^{2+}]_e$ did, or did not, induce release of Ca^{2+} from intracellular stores (e.g. capacitative Ca^{2+} entry; Wu et al., 1999), or alternatively, if pharmacological induction of intracellular Ca^{2+} release contributed to this potentiation. Therefore, in Ca^{2+}-free buffer, thapsigargin (1 μM) and the $mGlu_{1/5}$ agonist DHPG (100 μM) were employed to induce intracellular Ca^{2+} release (Figure 3.4E). However, only thapsigargin (1 μM) was effective in increasing $[Ca^{2+}]_i$, producing an increase of approximately 18% above basal $[Ca^{2+}]_i$ ($P < 0.05$, one-way ANOVA, *post hoc* test), compared to DHPG which produced a decrease of 15% below basal $[Ca^{2+}]_i$ ($P < 0.05$, one-way ANOVA, *post hoc* test) when averaged across the 5 min treatment period.

Considering that in the presence of Ca^{2+}, L-type Ca^{2+} channel blockers and the A_{2A} receptor antagonist ZM241385 had inhibited the $mGlu_{2/3}$-mediated potentiation of cAMP production (Table 2), the effect of these treatments on $[Ca^{2+}]_i$ was investigated (Figure 3.4F). While ZM241385 (1 μM) had little effect on $[Ca^{2+}]_i$ compared to 10 μM forskolin plus 100 nM LY379268 ($P > 0.05$, one-way ANOVA, *post hoc* test), an increase in $[Ca^{2+}]_i$ was evident in the presence of the L-type Ca^{2+} channel blockers ($P < 0.05$, one-way ANOVA, *post hoc* test). These blockers were included in the wash buffer (containing 1.8 mM Ca^{2+}, as per the cAMP assay), therefore while it appears that L-type Ca^{2+} channel blockers have increased $[Ca^{2+}]_i$ following

treatment with forskolin and LY379268, this effect is standardised to the already decreased $[Ca^{2+}]_i$ following pre-incubation with the blockers (see Figure 3.4D). LY379268 (100 nM) alone did not affect basal $[Ca^{2+}]_i$ in Ca^{2+}-free or 1.8 mM Ca^{2+} buffers (both conditions: $P > 0.05$, one-way ANOVA; not shown). These results suggest that in the presence of forskolin, agonists of $mGlu_{2/3}$ are capable of inducing intracellular Ca^{2+} release.

3.3.6. *$mGlu_{2/3}$ potentiation of cAMP production in astrocytes in the presence of additional stimulants*

Apart from forskolin, other receptor agonists were employed to induce cAMP production in astrocytes (Table 3.3). The non-selective β-adrenoceptor agonist isoprenaline (10 μM) and the non-selective adenosine agonist NECA (10 μM) both stimulated cAMP production to approximately equivalent levels in Ca^{2+}-containing buffer. However LY379268-induced potentiation of cAMP production only occurred during isoprenaline stimulus (224% of isoprenaline control; $P = 0.022$, *t*-test). In Ca^{2+}-free buffer, 100 nM LY379268 inhibited cAMP production stimulated by isoprenaline to 49% of control ($P = 0.0022$, *t*-test) and NECA to 21% of control. However due to the latter agonist only weakly stimulating cAMP production in Ca^{2+}-free buffer, the LY379268-induced inhibition failed to reach statistical significance (Table 3.3; $P > 0.05$, *t*-test). Evidence suggests that stimulation of the intracellular PLC/IP_3 pathway via $mGlu_{1/5}$ may be linked with intracellular effects induced by $mGlu_{2/3}$ (Schoepp et al., 1996a; Cormier et al., 2001) such that DHPG and 2*R*,4*R*-APDC act in synergy to increase cAMP production for example. However, in the present study 100 μM DHPG not only failed to induce cAMP production, but also failed to facilitate cAMP production when co-applied with 100 nM LY379268 (Table 3; $P > 0.05$, *t*-test) and 1 μM ($P > 0.05$, *t*-test; not

Figure 3.4 Pharmacological manipulation of $[Ca^{2+}]_i$ in neurons (A-C) and astrocytes (D-F).

A. In the presence of 1.8 mM Ca^{2+}, 50 mM KCl induces an increase in $[Ca^{2+}]_i$ (‡ $P < 0.05$, one-way ANOVA, *post-hoc* test). In the absence of 1.8 mM Ca^{2+}, 50 mM KCl is unable to induce increases in $[Ca^{2+}]_i$ ($P > 0.05$, one-way ANOVA, *post-hoc* test). Values represent the mean ± S.E.M. (n = 8) over a period of 5 min at 37°C.

B. Treatment of neurons in Ca^{2+}-free buffer with 10 μM forskolin decreased $[Ca^{2+}]_i$ slightly compared to basal, however treatment with both forskolin plus 100 nM LY379268 slightly increased $[Ca^{2+}]_i$ compared to basal.

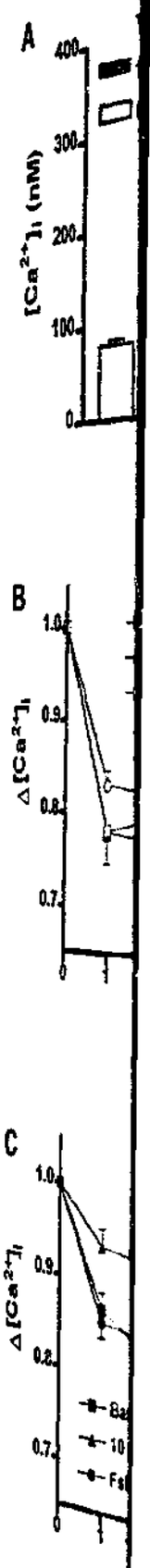
C. In Ca^{2+}-containing buffer, treatment of neurons with 10 μM forskolin increased $[Ca^{2+}]_i$ compared to basal values (*$P < 0.05$, one-way ANOVA, *post hoc* test). This increase was inhibited when co-treated with 100 nM LY379268 (#$P < 0.05$, one-way ANOVA, *post hoc* test).

D. In the absence of 1.8 mM Ca^{2+}, 50 mM KCl is unable to induce increases in $[Ca^{2+}]_i$. Values represent the mean ± S.E.M. (n = 8) over a period of 5 min at 37°C. Ca^{2+} channel blockers include 1μM nifedipine and 1 μM nimodipine and reduced the $[Ca^{2+}]_i$ by approximately 75% compared to 1.8 mM Ca^{2+} buffer. Ca^{2+} channel blockers included in the wash buffer also. In Ca^{2+}-containing buffer the increase in $[Ca^{2+}]_i$ was not statistically significant due to variability. In the absence of 1.8 mM Ca^{2+}, 50 mM KCl is unable to induce increases in $[Ca^{2+}]_i$ ($P > 0.05$, one-way ANOVA, *post-hoc* test).

E. Thapsigargin (1 μM), but not DHPG (100 μM) produces an increase in $[Ca^{2+}]_i$ in astrocytes when co-treated with 10 μM forskolin and 100 nM LY379268 in Ca^{2+}-free buffer. * $P < 0.05$ compared to basal, two-way ANOVA, *post hoc* test. # $P < 0.05$ compared to forskolin, two-way ANOVA, *post hoc* test.

F. Ca^{2+} channel blockers but not ZM241385 (1 μM) produce an increase in $[Ca^{2+}]_i$ in astrocytes when co-treated with 10 μM forskolin and 100 nM LY379268 in Ca^{2+}-containing buffer. However, the increase produced by the Ca^{2+} channel blockers here follows the already decreased $[Ca^{2+}]_i$ produced by the blockers prior to the addition of forskolin and LY379268 (as seen in A).

† $P < 0.05$ compared to treatment with both forskolin and LY379268, two-way ANOVA, *post hoc* test. LY379268 alone (with IBMX) had no significant effect on $[Ca^{2+}]_i$ compared to basal in either Ca^{2+}-free or 1.8 mM Ca^{2+} buffer (both conditions: $P > 0.05$, two-way ANOVA, *post hoc* test). In B, C, E and F all $[Ca^{2+}]_i$ are expressed as a ratio of the $[Ca^{2+}]_i$ prior to the addition of 10 μM forskolin and 100 nM LY379268 to give change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$).



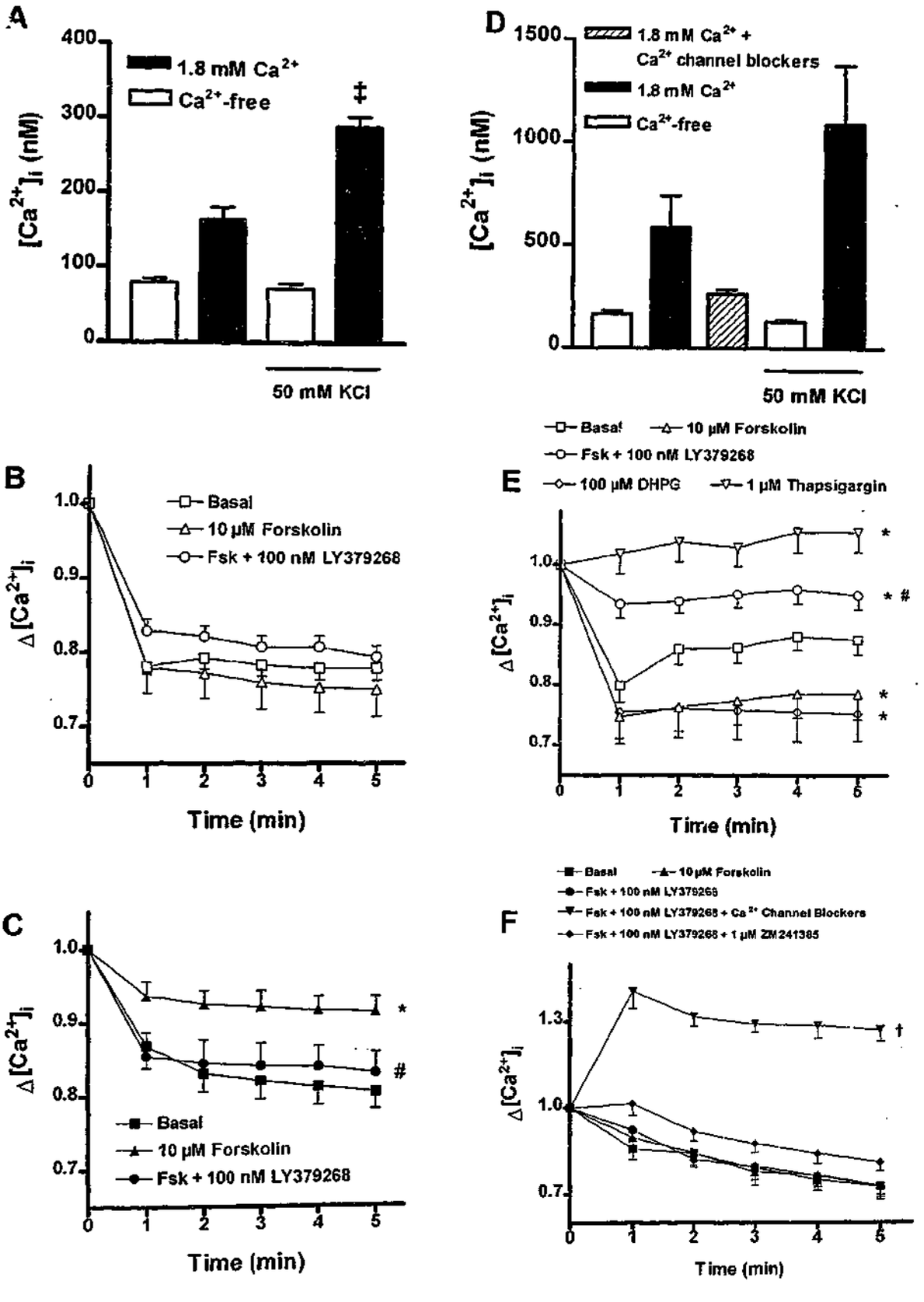

A
400
300
200
100
0
$[Ca^{2+}]_i$ (nM)
1.8 mM Ca^{2+}
Ca^{2+}-free
‡
50 mM KCl
D
1500
1000
500
0
$[Ca^{2+}]_i$ (nM)
1.8 mM Ca^{2+} + Ca^{2+} channel blockers
1.8 mM Ca^{2+}
Ca^{2+}-free
50 mM KCl
B
1.0
0.9
0.8
0.7
$\Delta[Ca^{2+}]_i$
Basal
10 µM Forskolin
Fsk + 100 nM LY379268
0 1 2 3 4 5
Time (min)
E
Basal
10 µM Forskolin
Fsk + 100 nM LY379268
100 µM DHPG
1 µM Thapsigargin
1.0
0.9
0.8
0.7
$\Delta[Ca^{2+}]_i$
* * # * *
0 1 2 3 4 5
Time (min)
C
1.0
0.9
0.8
0.7
$\Delta[Ca^{2+}]_i$
* #
Basal
10 µM Forskolin
Fsk + 100 nM LY379268
0 1 2 3 4 5
Time (min)
F
Basal
10 µM Forskolin
Fsk + 100 nM LY379268
Fsk + 100 nM LY379268 + Ca^{2+} Channel Blockers
Fsk + 100 nM LY379268 + 1 µM ZM241385
1.3
1.0
0.7
$\Delta[Ca^{2+}]_i$
†
0 1 2 3 4 5
Time (min)

Table 3.3 Ca^{2+}-dependent, $mGlu_{2/3}$-mediated potentiation or inhibition of cAMP production using various stimulants.

	cAMP Production in Astrocytes (pmol/mg protein/min)	
	Stimulant	Stimulant + 100 nM LY379268 (% of stimulant)
+ 1.8 mM Ca^{2+}		
10 µM Forskolin	10.1 ± 1.9	24.7 ± 5.2 (244)#
10 µM Isoprenaline	17.4 ± 1.1	39.1 ± 5.2 (224)*
10 µM NECA	18.4 ± 1.9	13.4 ± 3.1 (72)
100 µM DHPG	1.1 ± 0.1	1.4 ± 0.2 (127)
10 µM 1,9-Dideoxyforskolin	0.3 ± 0.1	0.1 ± 0.1
Ca^{2+}-free		
10 µM Forskolin	8.3 ± 0.3	5.0 ± 0.4 (58)#
10 µM Isoprenaline	21.2 ± 4.1	10.3 ± 1.5 (49)*
10 µM NECA	2.9 ± 1.2	0.6 ± 0.1 (21)
100 µM DHPG	0.2 ± 0.1	0.1 ± 0.0

Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, and values adjusted to reflect cAMP production per min. 10 µM 1,9-dideoxyforskolin and 100 µM DHPG were without effect in inducing cAMP formation, and no significant potentiation was observed when co-treated with 100 nM LY379268. 100 nM LY379268 was without effect in potentiating 10 µM NECA-induced cAMP production. # $P < 0.05$ compared to forskolin, as determined in section 3.2. * $P < 0.05$ compared to respective stimulant alone (*t*-test). n = 6-9.

shown). Finally, in the presence of Ca^{2+} (1.8 mM) and the inactive forskolin analogue 1,9-dideoxyforskolin (10 μM), LY379268 failed to increase cAMP (1-1000 nM; only 100 nM shown in Table 3.3), indicating that in these astrocytes LY379268 is unlikely to be mediating a potentiation effect though a G_s protein-coupled $mGlu_{2/3}$ or as a result of secondary effects of forskolin (Hoshi et al., 1988).

3.3.7. *CaMKII and agonist-induced cAMP production*

Further experiments were undertaken to determine the site of action of KN-62 which appeared to "restore" the LY379268-induced inhibition of cAMP production despite the presence of 1.8 mM Ca^{2+}. Pre-incubation with KN-62 (10 μM) failed to prevent the inhibition of forskolin-stimulated cAMP production produced by LY379268 (100 nM) in Ca^{2+}-free buffer ($P > 0.05$ compared to 10 μM forskolin plus 100 nM LY379268, *t*-test; Figure 3.5A). In Ca^{2+}-containing buffer, KN-62 (10 μM) inhibited the production of cAMP induced by 10 μM NECA by approximately 90% ($P = 0.0286$ compared to 10 μM NECA alone, *t*-test; Figure 3.5B), but not cAMP production induced by 10 μM isoprenaline ($P > 0.05$ compared to 10 μM isoprenaline alone, *t*-test; Figure 3.5B). These results suggest that stimulated CaMKII acts to promote the adenosine-activated A_{2A}-mediated potentiation of cAMP production, and is relatively specific for those responses induced by A_{2A} coupled to G_s over β-adrenoceptors coupled to G_s or $mGlu_{2/3}$ coupled to G_i.

3.4. Discussion

Group II mGlu receptors localised on neurones undoubtedly play an important role in the regulation of synaptic transmission, especially through their ability to regulate presynaptic neurotransmitter release (Cartmell and Schoepp, 2000). Other evidence emphasises that

Figure 3.5 Inhibition of CaMKII reduces NECA-induced production of cAMP.

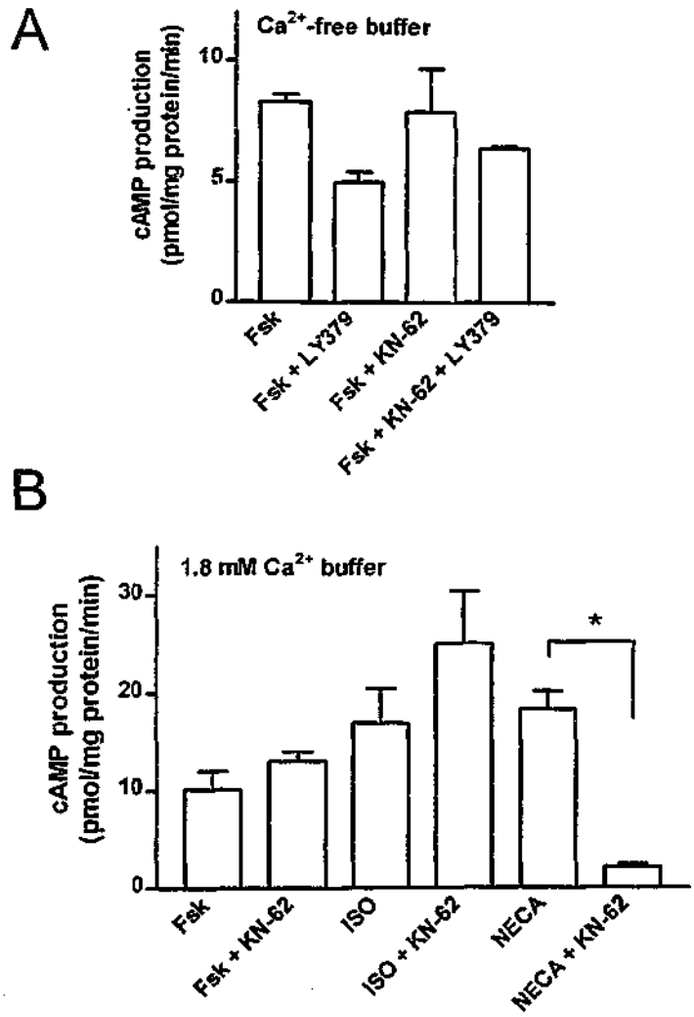
A. In Ca^{2+}-free buffer, KN-62 (10 μM) did not affect the ability of 100 nM LY379268 (LY379) to inhibit 10 μM forskolin (Fsk)-stimulated production of cAMP when compared to 10 μM forskolin plus 100 nM LY379268 in the absence of KN-62.

B. In 1.8 mM Ca^{2+} buffer, pre-incubation of KN-62 (10 μM) did not affect 10 μM forskolin (Fsk)- or 10 μM isoprenaline (ISO)-stimulated production of cAMP. In contrast, under similar treatment conditions, KN-62 did inhibit NECA-stimulated production of cAMP ($P = 0.0286$ compared to 10 μM NECA alone, *t*-test). Treatments were performed for 5 min at 37°C, standardised against the protein content of the sample, and values adjusted to reflect cAMP production per min.

* $P = 0.0286$ between indicated treatments, *t*-test. n = 6 – 9.

nM LY379268
ompared to 10

affect 10 μM
contrast, under
MP ($P = 0.0286$
min at 37°C,
o reflect cAMP



$mGlu_{2/3}$ located on glia are likely to contribute to cellular signalling (Winder and Conn, 1996) and neuroprotection (Bruno et al., 1998a; D'Onofrio et al., 2001). Classically, $mGlu_{2/3}$ have been defined, not only by their pharmacology but also by inhibition of stimulated production of cAMP. In this context our study has shown that 2*R*,4*R*-APDC and the more recently developed, potent and selective $mGlu_{2/3}$ agonist, LY379268, inhibit forskolin-stimulated production of cAMP in a PTX-sensitive and concentration-dependent manner in cortical and striatal neurones. While 2*R*,4*R*-APDC and LY379268 inhibited stimulated cAMP production in astrocytes under Ca^{2+}-free conditions, these agonists also potentiated stimulated cAMP production in the presence of physiological concentrations of calcium, an effect absent in cortical and striatal neurones. Furthermore, this study demonstrated that the potentiation of stimulated cAMP production mediated by $mGlu_{2/3}$ is regulated by the effective $[Ca^{2+}]_i$, which is sensitive to Ca^{2+} influx from the extracellular milieu and/or from mobilisation of intracellular Ca^{2+} stores. Based on an extensive range of pharmacological investigations targeting cellular signalling mechanisms, evidence was found suggesting that $mGlu_{2/3}$ mediated potentiation of cAMP production in astrocytes was associated with the PLC/IP_3 pathway, CaMKII and adenosine receptors.

The abilities of LY379268 and 2*R*,4*R*-APDC to inhibit and potentiate cAMP production in neurones and/or astrocytes correlate with the potencies found previously in cell lines (Monn et al., 1999). Since many early studies used non-selective $mGlu_{2/3}$ agonists, the resultant literature contains a panoply of interpretations including the involvement of $mGlu_{1/5}$ (Casabona et al., 1992; Schoepp et al., 1996a), possible group II-like mGlu receptors (Winder and Conn, 1995) and the involvement of other G protein-coupled receptors, including β-adrenoceptors (Balázs et al., 1998a,b), histamine (Selbie and Hill, 1998) and adenosine

receptors (Cartmell et al., 1993; Schoepp et al., 1996a; Ogata et al., 1996; Ribeiro, 1999; Cormier et al., 2001).

Protein and mRNA expression of the group I mGlu receptor subtype, $mGlu_5$, and the group II mGlu receptor subtype, $mGlu_3$, is understood to be localised on astrocytes *in vivo*, or cultured in the presence or absence of serum (Tamaru et al., 2001; Janssens and Lesage, 2001). Cultured cortical neurones express all subtypes of group I and II mGlu receptors (Janssens and Lesage, 2001). In agreement with earlier evidence (Cartmell et al., 1993), Schoepp et al. (1996a) found evidence for synergism between group I and II mGlu receptors, whereby increasing concentrations of 2*R*,4*R*-APDC increased cAMP formation in rat neonatal hippocampal slices only in the presence of DHPG, and vice versa, by a mechanism which involved endogenous adenosine release. Whilst no group I/II mGlu receptor synergism in astrocyte cultures was found in the present study, evidence existed for endogenous adenosine release such that adenosine deaminase or antagonism of A_{2A} receptors prevented the potentiation of cAMP production mediated by $mGlu_{2/3}$. Winder and Conn (1995) found that DCG-IV and L-CCG-I potentiated β-adrenergic stimulated cAMP production in adult rat hippocampal slices, in agreement with our current findings with LY379268 plus isoprenaline. However, in the present study $mGlu_{2/3}$ was able to inhibit β-adrenoceptor production of cAMP in the absence of extracellular Ca^{2+}. These results suggest that the potentiation reported briefly by Winder and Conn (1995) was most possibly a result of endogenous adenosine release produced by activation of $mGlu_{2/3}$ on astrocytes in the presence of sufficient $[Ca^{2+}]_i$ and G_s protein-coupled receptor stimulation and/or positive adenylate cyclase activity.

While our research had previously noted the sensitivity of cAMP signalling to extracellular Ca^{2+} (Moldrich and Beart, 2002), the present study sought to investigate this phenomenon in greater depth. The first observation made was that this Ca^{2+} sensitivity

occurred in forebrain astrocytes and not cortical or striatal neurones. Thus, the physiological concentrations of extracellular Ca^{2+} (1.8 mM) were replicated in astrocytes *in vitro* and demonstrated that the potentiation of cAMP production was dependent on the $[Ca^{2+}]_e$. Since these observations did little to explain the mechanisms involved, it was important to correlate this $[Ca^{2+}]_e$ with $[Ca^{2+}]_i$, and finally, to include treatments used to first characterise cAMP production in culture. What these studies collectively showed was that cAMP potentiation was dependent upon $[Ca^{2+}]_i$ and that this was likely due in part to Ca^{2+} influx which could be manipulated by adjusting the $[Ca^{2+}]_e$. Further evidence for this latter hypothesis came from employing L-type Ca^{2+} channel blockers to prevent Ca^{2+} influx. While astrocytes have been shown to express various Ca^{2+} channels (Verkhratsky and Steinhäuser, 2000), initial experiments showed that the L-type Ca^{2+} channel blockers, nifedipine and nimodipine, could inhibit $[Ca^{2+}]_i$ increases by approximately 75% in the presence of 1.8 mM Ca^{2+}. Consequently, these blockers were then employed to better understand the mechanisms behind the $mGlu_{2/3}$-mediated potentiation of cAMP production. Given the rapid nature of both Ca^{2+} influx and second messenger signalling, the L-type Ca^{2+} channels blockers were included in the wash buffers to pre-empt any Ca^{2+} influx. Ultimately, these results suggested that Ca^{2+} influx in astrocytes through predominantly L-type Ca^{2+} channels contributed to an increase in $[Ca^{2+}]_i$ which correlated with a $mGlu_{2/3}$-mediated potentiation of forskolin-stimulated cAMP production.

Sarco-endoplasmic reticulum Ca^{2+}-ATPases are transporters that actively sequester Ca^{2+} within the endoplasmic reticulum or to one of its sub-compartments. Thapsigargin acts indirectly by inhibiting these transporters and consequently preventing the counterbalancing of the passive Ca^{2+} leak from the stores to the cytosol (Treiman et al., 1998). Given the obvious sensitivity of the $mGlu_{2/3}$-mediated modulation of cAMP production to extracellular Ca^{2+},

thapsigargin was employed in this study to increase cytosolic $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. The results show that thapsigargin prevented the decrease in $[Ca^{2+}]_i$ induced by treatment with both forskolin plus LY379268 in astrocytes, and under similar conditions, facilitated the potentiation of forskolin-stimulated production of cAMP following activation of $mGlu_{2/3}$. Therefore, the present study not only linked the effect of $[Ca^{2+}]_e$ on $[Ca^{2+}]_i$ to the potentiation of cAMP production, but also demonstrated that in Ca^{2+}-free conditions mobilisation of intracellular Ca^{2+} stores increased $[Ca^{2+}]_i$ and that both mechanisms were likely to be operative in regulating $mGlu_{2/3}$ signalling.

In light of the evidence indicating adenosine release, it is possible that an increase in $[Ca^{2+}]_i$ results in activation of synaptotagmins, the putative targets of which include SNARE, syntaxin and SNAP-25 - key proteins expressed in astrocytes and involved in exocytosis (Sugita and Sudhof, 2000; Araque et al., 2000). Other possible targets of this increase in $[Ca^{2+}]_i$ could be AC isoforms - at the micromolar concentrations achieved here, intracellular Ca^{2+} is known to inhibit some negatively coupled AC isoforms (e.g. AC V and VI), which presumably transduce the $mGlu_{2/3}$-G_i protein signal in astrocytes (Charbardès et al., 1999). Alternatively, or simultaneously, such increases in $[Ca^{2+}]_i$ could stimulate other positively coupled AC isoforms through calmodulin binding (e.g. AC I and VIII) which could transduce β-adrenergic/A_{2A}-G_s protein signals (Antoni, 2000), or through stimulating PKC activity (Defer et al., 2000).

In neurones, CaMKII has been known for some time to be directly activated by autophosphorylation and/or from its interaction with cytosolic Ca^{2+}/calmodulin complexes. Although, in neurones, regulation of CaMKII is important in synaptic transmission, synaptic plasticity and epileptogenesis (Lowenstein 1996; Bronstein et al., 1993), little is known about the functions or isoforms of CaMKII in astrocytes. CaMKII has been shown to be capable of phosphorylating AC III as a result of an increase in $[Ca^{2+}]_i$ thereby inhibiting AC III-mediated

production of cAMP (Wei et al., 1996). Thus, in the present system, CaMKII may act at AC III to inhibit the secondary adenosine-induced rise in cAMP production. However, the observation that KN-62 inhibited NECA-stimulated cAMP production (but did not modulate forskolin- or β-adrenoceptor-stimulated cAMP production) suggests that CaMKII is most likely involved in inhibiting adenosine receptor-induced cAMP production at the site of the receptor itself, the G protein or a specifically coupled AC. Clearly, the role of CaMKII in astrocytes needs to be studied in some detail with attention focusing on its interfaces with cellular signaling cascades.

Forskolin and cAMP analogues are known to inhibit the function of presynaptic group II mGlu receptors in area CA3 of the hippocampus (Kamiya and Yamamoto, 1997; Maccaferri et al., 1998). Additional research showed that the function and coupling of $mGlu_2$ to G proteins could be restored by inhibitors of PKA (Schaffhauser et al., 2000) whereby Ser^{843} was identified as the primary site of PKA-induced phosphorylation. $mGlu_3$ contains a similar PKA consensus site at Ser^{845}. Given that in our system one might expect to induce PKA-mediated phosphorylation, the PKA inhibitor H89 was employed to determine if the LY379268-induced cAMP response was sensitive to phosphorylation. Since H89 obviously increased the level of forskolin-stimulated cAMP production, there exists the potential for the interaction of cAMP with PKA in these astrocytes. Interestingly, stimulation of cAMP production by forskolin is either not able to inhibit the coupling of $mGlu_3$ to G_i via PKA-induced phosphorylation, or alternatively PKA-induced phosphorylation is not adequate to arrest the gross $mGlu_3$-mediated signal transduction. Because quite simply, in our study and many others, $mGlu_{2/3}$ agonists are capable of reducing stimulated cAMP production, demonstrating successful G_i protein coupling. Indeed, LY379268 was still able to potentiate cAMP production with the same efficiency (compared to 100% forskolin plus H89 control) despite the presence of H89.

The present study also demonstrated the importance of PLC in the $mGlu_{2/3}$-mediated potentiation of cAMP production, and in light of other evidence in this study, recruitment of PLC presumably results in the release of Ca^{2+} from intracellular stores. Since the $mGlu_{1/5}$ agonist DHPG failed to facilitate potentiation of cAMP in the present study it is unlikely this receptor-induced recruitment of the PLC/IP_3 pathway and subsequent intracellular Ca^{2+} release from IP_3-sensitive stores is responsible for the potentiation observed. While generally, G_i protein-coupled A_1 receptors decrease cAMP production (Ralevic and Burnstock, 1998), they have also been shown to potentiate the intracellular effects mediated by $mGlu_5$, presumably by synergy with the PLC/IP_3 pathway (Cormier et al., 2001). However, the G_s protein-coupled A_{2A} receptor antagonist ZM241385 inhibited the potentiation of cAMP observed in the present study which involved the PLC/IP_3 pathway. Following G protein activation, βγ subunit release has been shown in other systems to stimulate G_s-associated AC II (Antoni, 2000) while increasing evidence links βγ subunit release with activation of IP_3 kinase and PLC. A recent elegant study showed how IP_3 kinase was involved in neuroprotection induced by LY379268 and mediated through astrocytes (D'Onofrio et al, 2001). However, $mGlu_5$ activation by DHPG which is known to involve PLC, did not induce potentiation of cAMP in the present study, and was similarly absent in the study by Balázs et al. (1998a). Consequently, in the astrocytes cultured here it is unlikely that $mGlu_5$-dependent G protein-coupled receptor (GPCR) cross-talk contributes significantly to the potentiation observed, unlike that seen in hippocampal astrocytes (Cormier et al., 2001) or in neurones (Ciruela et al., 2001), indicating possible regional and cell-type specific patterns of GPCR interactions. GPCR pathways and AC signalling have been discussed in more detail elsewhere (Selbie and Hill, 1998; Ford et al., 1998; Tesmer and Sprang, 1998; Antoni, 2000; Fimia and

Sassone-Corsi, 2000). Figure 3.6 represents some signalling pathways in astrocytes implicated in the results arising from the present study.

Adenosine has been shown to be neuroprotective in numerous models of neuronal, glial and *in vivo* injury via inhibiting NO toxicity and Ca^{2+} influx in neurones, or in glial cells because of its regulatory role on the Ca^{2+}- and cAMP-dependent intracellular signalling which influences cellular proliferation and differentiation, and affects intercellular signalling involving microglia (Schubert et al., 1996, 1997, 2001). For example, A_1 receptor-mediated Ca^{2+} signalling has been shown to cause a prolonged potentiation of the A_2 receptor-mediated intracellular cAMP rise leading to increased expression of K^+ and Cl^- channels (Verkhratsky and Steinhäuser, 2000), and of glutamate transporters (Eng et al., 1997; Schlag et al., 1998). The putative actions of cAMP to promote the increased expression of glial glutamate transporters EAAT1 and EAAT2 might be important in preventing excitotoxicity since they are the predominant transporters responsible for the clearance of extracellular glutamate (Danbolt, 2001). Alternatively, A_1 and $mGlu_3$ co-stimulation induces protein synthesis and trophic factor release, which subsequently protects neurones against toxic insult (Ciccarelli et al., 1999). Finally, nucleoside transport of adenosine and its metabolised analogues has been shown to be important in the survival of glia (Jurkowitz et al., 1998).

Little is known about the expression and function of many key intracellular signals in astrocytes. For example, knowledge of the expression and coupling of AC isoforms would help explain the interaction of GPCR second messengers and indicate the sensitivity of these isoforms to Ca^{2+} and/or calmodulin. Stimulated PKC may also alter the ability of the various AC isoforms to integrate inputs in these astrocytes (Jacobowitz et al., 1993). The possibility that other cAMP-dependent kinases or guanine nucleotide exchange factors are involved in the responses observed should not be discounted, especially in light of the recent discovery of

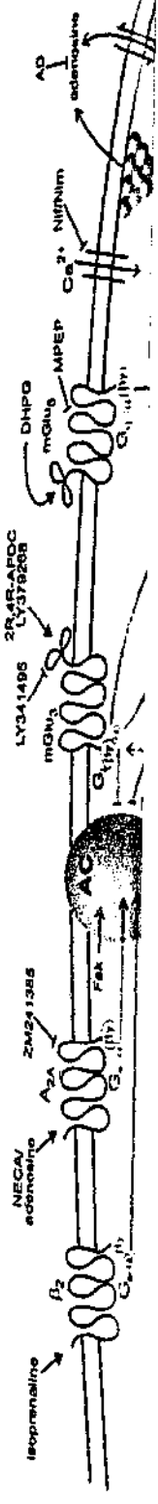
Sassone-Corsi, 2000). Figure 3.6 represents some signalling pathways in astrocytes implicated in the results arising from the present study.

Adenosine has been shown to be neuroprotective in numerous models of neuronal, glial and *in vivo* injury via inhibiting NO toxicity and Ca^{2+} influx in neurones, or in glial cells because of its regulatory role on the Ca^{2+}- and cAMP-dependent intracellular signalling which influences cellular proliferation and differentiation, and affects intercellular signalling involving microglia (Schubert et al., 1996, 1997, 2001). For example, A_1 receptor-mediated Ca^{2+} signalling has been shown to cause a prolonged potentiation of the A_2 receptor-mediated intracellular cAMP rise leading to increased expression of K^+ and Cl^- channels (Verkhratsky and Steinhäuser, 2000), and of glutamate transporters (Eng et al., 1997; Schlag et al., 1998). The putative actions of cAMP to promote the increased expression of glial glutamate transporters EAAT1 and EAAT2 might be important in preventing excitotoxicity since they are the predominant transporters responsible for the clearance of extracellular glutamate (Danbolt, 2001). Alternatively, A_1 and $mGlu_3$ co-stimulation induces protein synthesis and trophic factor release, which subsequently protects neurones against toxic insult (Ciccarelli et al., 1999). Finally, nucleoside transport of adenosine and its metabolised analogues has been shown to be important in the survival of glia (Jurkowitz et al., 1998).

Little is known about the expression and function of many key intracellular signals in astrocytes. For example, knowledge of the expression and coupling of AC isoforms would help explain the interaction of GPCR second messengers and indicate the sensitivity of these isoforms to Ca^{2+} and/or calmodulin. Stimulated PKC may also alter the ability of the various AC isoforms to integrate inputs in these astrocytes (Jacobowitz et al., 1993). The possibility that other cAMP-dependent kinases or guanine nucleotide exchange factors are involved in the responses observed should not be discounted, especially in light of the recent discovery of

Figure 3.6. Model of putative, key signalling pathways in astrocytes implicated from previous findings and those from the present study.

Arrows represent activation. Lines with T-shaped ends indicate inhibition. Broken lines indicate indeterminate pathways or pathways involving multiple, unlabelled steps. Abbreviations not made in the text: AD, adenosine deaminase; CREB, cAMP-responsive-element-binding protein; Nif/Nim, nifedipine/nimodipine; MEK, MAP/ERK kinase; PIP_2, phosphatidylinositol(4,5)-biphosphate; IP_3, inositol 1,4,5-triphosphate; RyR, ryanodine receptor.



mplicated from

on. Broken lines

s. Abbreviations

element-binding

tidylinositol(4,5)-

Epac, a cAMP-dependent kinase with links to PI_3 kinase and PKB (Kawasaki et al., 1998; Mei et al., 2002). For example, activation of G_s protein-coupled β_2-adrenoceptors expressed in HEK-293 cells induced Ca^{2+} mobilisation and PLC activation via a guanine-nucleotide exchange factor and GTPase (Schmidt et al., 2001). Finally, the presence of putative functional complexes between GPCRs, as for example between $mGlu_{1a}$ and A_1 (Ciruela et al., 2001), needs to be determined.

This study has demonstrated a unique effect in astrocytes whereby potentiation of stimulated production of cAMP can be induced by agonists acting at $mGlu_{2/3}$ to promote adenosine release. Furthermore, this effect is dependent on $[Ca^{2+}]_i$ which is in turn influenced by extracellular and intracellular Ca^{2+} stores linked to PLC and CaMKII. Ultimately, our data supports existing evidence for elaborate pathways and receptor interactions amongst GPCRs in astrocytes that are likely to modulate synaptic activity, be it in the normal or pathological synaptic milieu.

CHAPTER 4

$mGLU_{2/3}$ AND EPILEPSY

4.1. Introduction

Excessive glutamatergic neurotransmission is understood to be one of the primary pathological mechanisms behind the aetiology of numerous types of epilepsy (Chapman et al., 1996). Consequently, attempts to modulate glutamatergic neurotransmission in animal models of epilepsy via activation of group II mGlu receptors have previously employed the non-selective agonists (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), (*S*)-4-carboxy-3-hydroxyphenylglycine (*S*-4C3HPG), (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I) and (2*S*,2'*R*,3'*R*)-2-(2',3'dicarboxycyclopropyl)glycine (DCG-IV) (Meldrum et al., 1996; Dalby and Thomsen, 1996; Attwell et al., 1998a,b). The agonists *S*-4C3HPG, L-CCG-I and 1*S*,3*R*-ACPD have been shown to inhibit sound-induced clonic seizures and possess variable activity inhibiting chemoconvulsant-induced clonic seizures (Meldrum et al., 1996; Dalby and Thomsen, 1996). The agonists 1*S*,3*R*-ACPD and DCG-IV have been shown to be inhibit electrically-stimulated seizures in amygdala-kindled rats via intra-amygdaloid injection (Attwell et al., 1998a; Suzuki et al., 1996), while DCG-IV also partly inhibits kainate-induced limbic seizures (Miyamoto et al., 1997). Anticonvulsant activity has also been reported for the more selective $mGlu_{2/3}$ agonist 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate (2*R*,4*R*-APDC) in amygdala-kindled rats (Attwell et al., 1998b). The highly selective $mGlu_{2/3}$ agonists (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), (–)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) and (–)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY389795) were originally reported to inhibit 1*S*,3*R*-ACPD-induced seizures in mice (Monn et al., 1997; Monn et al., 1999). While more recently, LY354740, which is less potent than LY379268 and LY389795, has been shown to

inhibit pentylenetetrazol- and picrotoxin-induced clonic convulsions (Klodzinska et al., 2000).

The present study investigated the anti-epileptic activity of 2*R*,4*R*-APDC, LY379268 and LY389795 against sound-induced clonic seizures in audiogenic mice and rats, DHPG-induced limbic seizures, absence seizures in lethargic mice and electrically-stimulated seizures in amygdala-kindled rats. The proconvulsant profile of the agonists, and the $mGlu_{2/3}$ antagonist LY341495 was also investigated.

4.2. Methods

All regulated procedures performed in this study were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 under project licence 70/4775 at the Institute of Psychiatry, King's College, London. A full list of materials used appears in Appendix IV.

4.2.1. *Sound-induced seizures in DBA/2 mice*

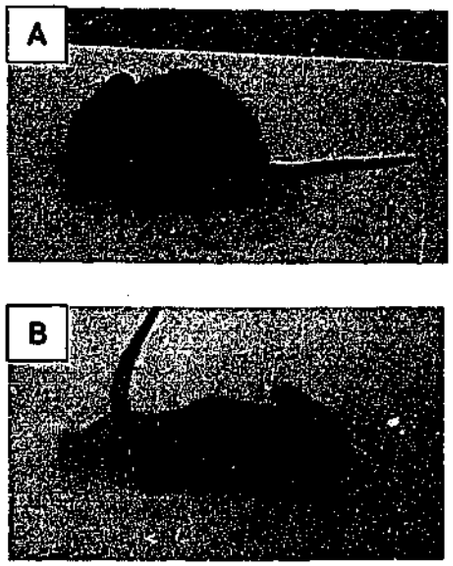
Dilute brown agouti (DBA/2) mice (male and female, age 21-28 days, 7-15g weight; Institute of Psychiatry colony) were weaned at 21 days, housed on a 12 h dark/12 h light cycle and allowed free access to food and water. The mice were randomly assigned to groups of 10 for experiments. DBA/2 mice undergo a well-characterised seizure response when exposed to loud sound stimulus which consistently results in whole body clonus (Chapman and Meldrum, 1987). Drug or vehicle were administered intracerebroventricularly (i.c.v.) (1 mm anterior to the bregma, 1 mm lateral to the midline, to a depth of 3 mm; Franklin and Paxinos, 1997) under light fluothane anaesthesia using a Hamilton syringe with a 25 short-gauge butterfly needle for delivering a volume of 10 μl.

Drug or vehicle was also administered intraperitoneally (i.p.) in a volume of 100 μl/10 g body weight. Following drug or vehicle injections, the mice were maintained at a body temperature of 36-38°C by applying heating lamps when required. Mice were observed for abnormal motor behaviour or proconvulsant effects of the drugs prior to testing for sound-induced seizures.

Anticonvulsant testing was carried out on individual mice 15–120 min following administration of drug or vehicle under a perspex dome (58 cm in diameter) fitted with a doorbell generating a sound of 110 dB for a period of 60 s or until the onset of clonic seizure. The sound stimulus produced a sequential seizure response, consisting of a wild running phase (1-4 s latency; score 1), clonic seizures (4-15 s latency; score 2), tonic extension (10-30 s latency; score 3) and occasionally respiratory arrest (20-40 s latency; score 4) (Figure 4.1; Chapman and Meldrum, 1987).

The agonists were dissolved in water and adjusted to pH 7.3-7.7 with NaOH for either i.c.v. or i.p. injection. 2*R*,4*R*-APDC, LY379268 or LY389795 (0.001–40 nmol i.c.v. or 1–100 mg/kg i.p., n = 10 per group) were injected 15 min prior (-15 min) to being tested for sound-induced seizures. A timecourse of action was determined for the agonists by testing groups of mice (n = 10) injected i.c.v. (0.3–20 nmol, 15–120 min) or i.p. (30 mg/kg, 15–30 min). A dose-response suppression of the inhibition of sound-induced seizures by LY379268 was established with the $mGlu_{2/3}$ antagonist (α*S*)-α-amino-[(1*S*,2*S*)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY341495; Kingston et al., 1998; 10–300 nmol, i.c.v.).

Figure 4.1. Photographs representing the sound-induced clonus of DBA/2 mice. A. Unstimulated mouse. B. Stimulated mouse with forelimb and hindlimb extension during clonus (Score 2).



4.2.2. *Rotarod performance in DBA/2 mice*

Drug-induced motor-impairment was assessed using a rotarod (Ugo Basile). Groups of DBA/2 mice (n = 10) were trained prior to drug administration to remain for 2 min on a rotating wooden dowel (diameter 28 mm), fitted with shallow grooves every 20° and rotating with a speed 20 rpm. Following administration of 2*R*,4*R*-APDC, LY379268 or LY389795 (-15 min, 0.01–40 nmol i.c.v. or 30 mg/kg i.p) the rotarod performance of the group of mice was assessed for 2 min by recording the time spent on the rotarod before falling off. The mean rotarod performance for each group was expressed as a percentage of the time spent on the rotarod before injection (2 min) and used for calculating the ED_{50} value for motor impairment.

4.2.3. Seizures induced by DHPG administration in DBA/2 mice

DBA/2 mice (n = 10 per group) received 1.5 μmol (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG) i.c.v. (pH 7.4) in a volume of 10 μl under light fluothane anaesthesia and were observed for the following 90 min for the occurrence of seizure-related activity. DHPG-induced *seizure activity* included hindlimb scratching, mouth and/or forelimb clonus, forelimb and hindlimb extension, rearing and falling, and head and tail extension. Quantification of DHPG-induced seizure activity was based on a score (DHPG-induced seizure score) whereby the occurrence of each of the above characteristics received one score. DHPG-induced *seizures* were characterised by forelimb and hindlimb extension, and/or head and tail extension. The agonists (0.001–40 nmol) were co-injected with DHPG, or alternatively, LY379268 or LY389795 (10 mg/kg, i.p.) were administered 50 min following the i.c.v. injection of DHPG to coincide with the DHPG-induced development of seizures, and observed until 90 min following the administration of DHPG.

4.2.4. Sound-induced seizures in Genetically Epilepsy Prone-9 (GEP) rats

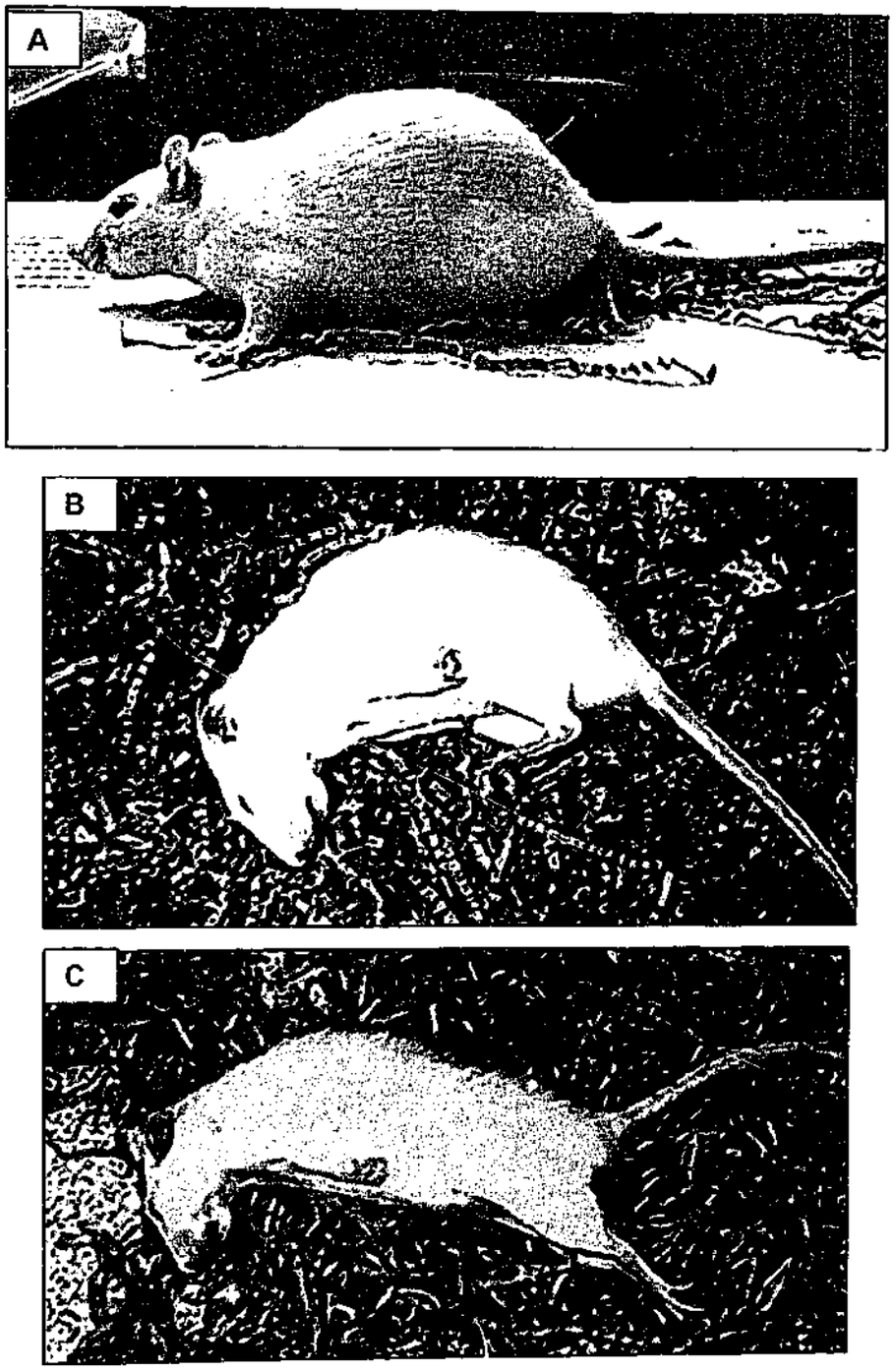
Adult GEP rats (Institute of Psychiatry colony) of either sex were housed in temperature and humidity controlled room on a 12 h light/12 h dark cycle and allowed free access to food and water. GEP rats were tested for sound-induced seizures on three consecutive days before treatment, only those animals which responded with three full seizures were included in the study. Testing was carried out in an enclosed chamber fitted with a doorbell generating a sound stimulus of 100 dB for a period of 60 s or until the onset of whole-body tonus (n = 4 per group). Scoring of sound-induced seizures in GEP rats was based on that of Jobe et al. (1973) whereby 0 = no response, 1 = wild running, 2-3 = partial whole-body clonus followed by one or two episodes of wild running, 4-5 = whole-body clonus with one or two episodes of wild running, 6-7 = whole-body clonus and partial tonic limb extension following one or two episodes of wild running, 8-9 = one or two episodes of wild running followed by whole-body clonus and tonic extension. Photos demonstrating GEP rats in typical stage 7 clonus and stage 9 tonus are presented in Figure 4.2. Vehicle (water) or the agonists were administered i.p. in a volume of 200 µl/200 g, 30 min or 1 h prior to exposure to sound stimulus.

4.2.5. Absence epilepsy in lethargic (lh/lh) mice

Lethargic (*lh/lh*) mice (Institute of Psychiatry colony) were bred and maintained as previously described (Chapman et al., 1999). A group of *lh/lh* mice of either sex from age of 8–12 weeks (20–25 g) were randomly selected for surgery (n = 5–6 per group).

The mice were anaesthetised with isoflurane-RM (4–4.5%) in medical oxygen (2.5 L/min) and immobilised in a stereotaxic holder. Bilateral burr holes were drilled over the frontal cortex (1.5 mm anterior to bregma and 1.5 mm lateral to the midsagittal suture;

Figure 4.2. Representative photos of GEP rats in various sound-induced seizure stages: A. Unstimulated GEP rat (Stage 0) **B**. Stimulated GEP rat undergoing clonus (stage 7), **C**. Stimulated GEP rat undergoing tonus (stage 9).



Franklin and Paxinos, 1997) and parietal cortex (left: 3 mm and right: 1 mm posterior to lambda, and 1.5 mm lateral to midsagittal sinus; Franklin and Paxinos, 1997) for stainless steel microelectrodes. Another burr hole was made 1 mm left of midsagittal sinus and 1 mm anterior to lambda for guide cannula (gauge 21) implantation (0.8 mm below dura). The implants were fixed with dental acrylic and epoxy resin. The electrodes were connected to a female plug for attachment to the Grass electroencephalogram (EEG) recorder.

Testing of the agonists (prepared as described in 2.1) began at least one week after surgery. During the EEG recording, the testing session was organised into 15 min epochs with the first epoch recording immediately prior to injection of vehicle or agonist (pre-injection epoch), and 6 epochs following injection. Injection cannulae (27 gauge) were lowered 2 mm beyond the edge of the guide cannula to the ventricle. The vehicle (water) or agonist (1 and 10 nmol) was infused at 2.5 μl/min in a total volume of 10 μl via a Hamilton syringe using a CMA/100 infusion pump. The injection cannula was withdrawn 1 min following infusion.

During each recording the behavioural changes after drug treatment in comparison to vehicle were noted. To minimise bias to observations associated with sedation, the *lh/lh* mouse was frequently aroused by a clapping noise (Hosford et al, 1992). The quantification of absence seizures was based on the duration (s) of EEG spike and wave discharges or polyspikes, as described by Hosford et al. (1992) (i.e. amplitude not less than 60 μV and frequency range of 5–6 Hz during seizures of not less than 0.6 s). The *lh/lh* mice spent on average approximately 1–2 min out of every 15 min pre-injection epoch in absence seizures according to these criteria. EEG recordings were made at an amplification of 200 μV/cm and a chart speed of 3 mm/s. To assess the pharmacological effect of the agonists the spike and wave discharge per epoch was calculated and expressed as a percentage of the spike and

wave discharge from the corresponding pre-injection epoch. In this way, the spike and wave discharge for each epoch following administration of the vehicle and agonists is presented as a mean ± S.E.M. of that treatment group (n = 5–6).

4.2.6. *Electrical stimulation of amygdala-kindled rats*

Male Wistar rats (Charles River, Margate, UK) weighing between 250–300 g at the time of surgery were housed in groups of four and allowed free access to food and water under a 14 h light/10 h dark cycle in an environment maintained at 19–22°C with a relative humidity of 55 ± 3%.

Animals were anaesthetised with 4–5% isoflurane-RM mixed with medical oxygen at 2.5 L/min and maintained in this way throughout surgery. The animals were placed in a stereotaxic frame and five burr holes were drilled into the cranium for the electrode assembly. Two cortical electrodes were implanted in the region of the sensorimotor cortex approximately 1.5 mm anterior to bregma, left and right of the intraural line. Earth and anchor electrodes were implanted in the region of the parietal cortex, left and right of the intraural line. A twisted bipolar, Teflon-coated, platinum/iridium electrode was implanted in the left basolateral amygdala (from interaural, AP +6.2 mm, L +5.0 mm and V +1.5 mm; Paxinos and Watson, 1997). The electrodes were connected to a female plug, for attachment to the EEG recorder, and the entire assembly fixed in place with dental acrylate and epoxy resin.

At least 7 days after surgery, animals were assessed for basal EEG activity. Afterdischarge spike and wave activity was induced using the following stimulation parameters: 2 s duration, 50 pulses/s, bipolar, 1 ms pulse-width and initially 1 V. Animals were repeatedly stimulated at 2 min intervals using 0.2 V increments on each stimulation

until an afterdischarge was achieved in the amygdala EEG trace. Once this threshold stimulation was determined, the animals were stimulated at this intensity once daily until fully kindled whereby stimulus of the threshold intensity produced five consecutive stage 5 seizures as described by Racine et al. (1972). Briefly, the animals experienced an electrically stimulated motor seizure when the threshold intensity produced spike and wave discharge (stage 1) followed by oral and facial automatisms (e.g. chewing, head nodding; stage 2), unilateral forelimb clonus (stage 3), bilateral forelimb clonus and rearing (stage 4), and rearing and falling (stage 5). Animals were electrically stimulated once, 24 h prior to drug administration to confirm the induction of stage 5 seizures. The agonists (3–30 mg/kg, i.p.) were administered 30 min prior to the first electrical stimulation, where a significant inhibition of seizure activity was observed a further stimulation 6 h following agonist injection was performed.

4.2.7. Statistics

ED_{50} values for the inhibition of sound-induced clonic seizures or DHPG-induced seizures, or IC_{50} values for the suppression of agonist inhibition of sound-induced seizures by LY341495 were calculated with 95% confidence limits according to the method of Litchfield and Wilcoxon (1949). ED_{50} values from rotarod testing of motor-impairment were similarly calculated.

Scores were established for sound-, DHPG- and electrically-induced seizure activity as described in sections 2.1 and 2.4 for sound stimulation of DBA/2 and GEP rats respectively, section 2.3 for the chemoconvulsant stimulus of DHPG, and section 2.6 for electrical stimulation of amygdala-kindled rats. Significant differences between the scores of treatment groups were determined using the Mann-Whitney U test or Wilcoxon Signed

Rank test whereby $P < 0.05$. The mean ± S.E.M. of the score per animal of a treatment group across the experimental models are presented.

Significant differences in the duration of the spike and wave discharge of *lh/lh* mice following treatment with the agonists were compared with the vehicle using a one-way analysis of variance (ANOVA) and Newman-Keuls *post hoc* comparison where $P < 0.05$. Comparisons in the duration of spike and wave discharge in electrically stimulated amygdala-kindled rats were made between the treatment and the -24 h control for the same animal, significant differences were determined using a paired Student t-test where $P < 0.05$. The duration of spike and wave discharge for treatments is expressed as the mean ± S.E.M. percentage of the -24 h control.

Proconvulsant activity following i.c.v. injection in DBA/2 mice was determined by recording behaviour different to that of the untreated mice and assigning a score of one per behaviour type. Significant statistical differences in the score between treatment groups were determined using the Mann-Whitney U test whereby $P < 0.05$. The mean ± S.E.M. score per animal in the treatment groups are presented.

4.3. Results

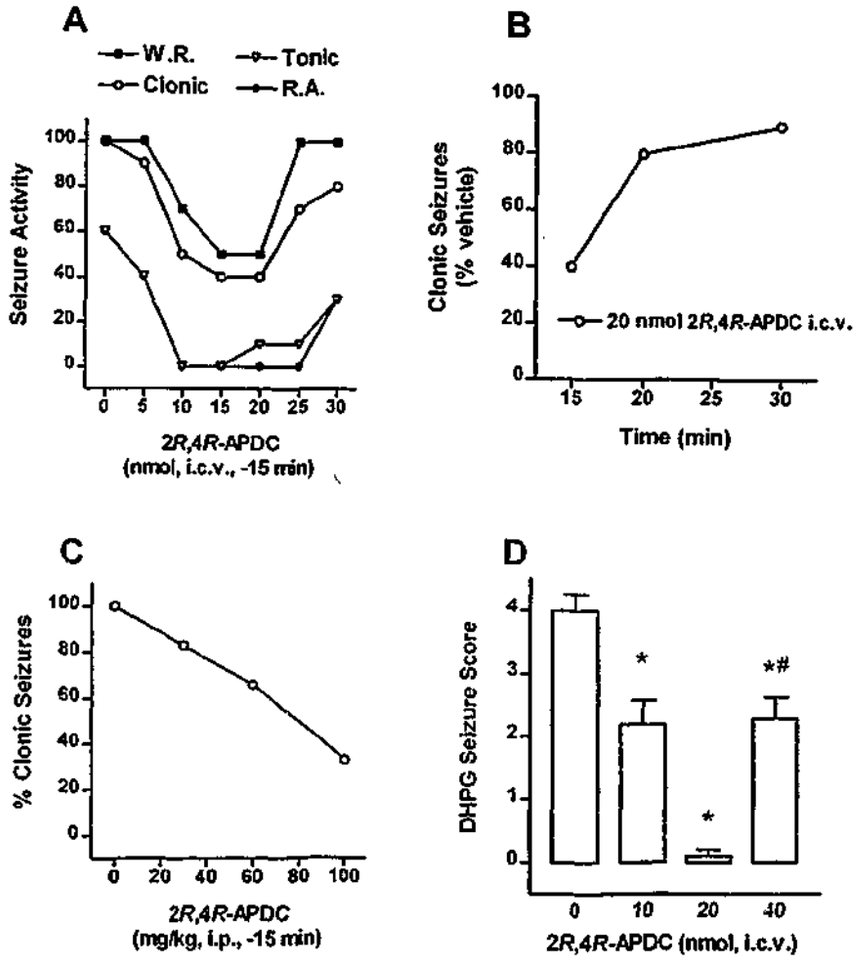
4.3.1. Inhibition of sound-induced seizures in DBA/2 mice

2*R*,4*R*-APDC (10-25 nmol, i.c.v.) produced a significant inhibition of the sound-induced seizure score in DBA/2 mice when compared to vehicle ($P \leq 0.0089$). However, at 30 nmol 2*R*,4*R*-APDC the inhibition of wild running, clonus, tonus and respiratory arrest had decreased compared to 15-20 nmol such that the score of DBA/2 mice at 30 nmol following sound stimulus was significantly greater than that at 15 and 20 nmol ($P = 0.0185$ and $P = 0.0288$ respectively). Inhibition of sound-induced seizure activity is presented in

Figure 4.3A. In the period following i.c.v. injection of 2*R*,4*R*-APDC and before exposure to sound stimulus, DBA/2 mice exhibited relatively normal behaviour at all doses tested without sedation or proconvulsant activity. The response of DBA/2 mice to sound-induced clonic seizures was also investigated at 20 min and 30 min following i.c.v. injection (Figure 4.3B). 2*R*,4*R*-APDC (20 nmol) exhibited a time-dependent loss of anticonvulsant activity whereby the score was not significantly different from that of vehicle within 20 min ($P = 0.3150$; Figure 4.3B). DBA/2 mice demonstrated relatively normal behaviour throughout the entire timecourse. The inhibition of sound-induced clonic seizures by 20 nmol 2*R*,4*R*-APDC was completely reversed by the $mGlu_{2/3}$ antagonist LY341495 (100 nmol; $P = 0.0101$). At 100 nmol (i.c.v., -15 min), LY341495 alone had no proconvulsant activity. 2*R*,4*R*-APDC inhibited sound-induced clonic seizures in a dose-dependent manner when administered systemically (30-100 mg/kg, i.p., -15 min; $P = 0.0411$ (100 mg/kg)), whereby ED_{50} = 75 [25-116] mg/kg (Figure 4.3C). A reversal of anticonvulsant activity was not observed over the doses tested. The behaviour of DBA/2 mice following i.p. administration of 2*R*,4*R*-APDC was not markedly different from that of vehicle.

Sound-induced seizures in DBA/2 mice were similarly suppressed in a dose-dependent manner 15 min following i.c.v. administration of LY379268 (0.001–0.3 nmol) and LY389795 (0.001–3 nmol) whereby ED_{50} = 0.08 [0.02–0.33] nmol, E_{max} = 0% of vehicle (0.3 nmol) and ED_{50} = 0.82 [0.27–3.24] nmol, E_{max} = 0% of vehicle (3 nmol) respectively (Figure 4.4A). Both agonists significantly reduced the score of sound-induced seizure activity ($P \leq 0.0288$). At higher doses (≥ 3 nmol) the agonists produced some hypolocomotion and sedation, however proconvulsant activity or hyperlocomotion was not observed (≤ 20 nmol, i.c.v.). LY379268 (0.3 nmol) and LY389795 (3 nmol) demonstrated a time-dependent loss of anticonvulsant activity, whereby the score of seizure activity 30 min

Figure 4.3 Anticonvulsant activity of 2*R*,4*R*-APDC against sound- and DHPG-induced seizures in DBA/2 mice.



A. Inhibition of seizure-related behaviour by 2*R*,4*R*-APDC (i.c.v., -15 min, $n = 10$). W.R.: wild running, R.A.: respiratory arrest.

B. Timecourse of 20 nmol 2*R*,4*R*-APDC inhibition of clonic seizures (i.c.v., $n = 10$).

C. Inhibition of clonic seizures by systemic administration of 2*R*,4*R*-APDC (i.p., -15 min, $n = 6$).

D. 2*R*,4*R*-APDC inhibition of DHPG-induced seizure activity (DHPG Seizure Score)

*$P < 0.05$ when compared to vehicle.

#$P < 0.05$ when compared to 20 nmol 2*R*,4*R*-APDC (Mann-Whitney U test; i.c.v., $n = 10$).

Figure 4.4. Inhibition of sound-induced seizures in DBA/2 mice following i.c.v. (A–C) or i.p. (D–E) administration of the LY379268 and LY389795.

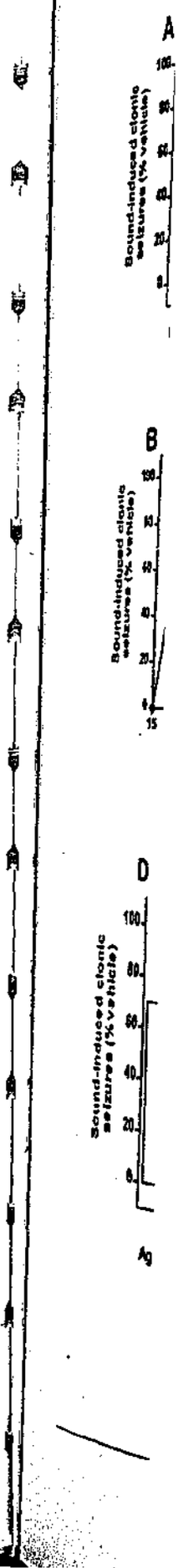
A. Dose-dependent (non-linear, variable-slope regression) inhibition of seizures by the agonists.

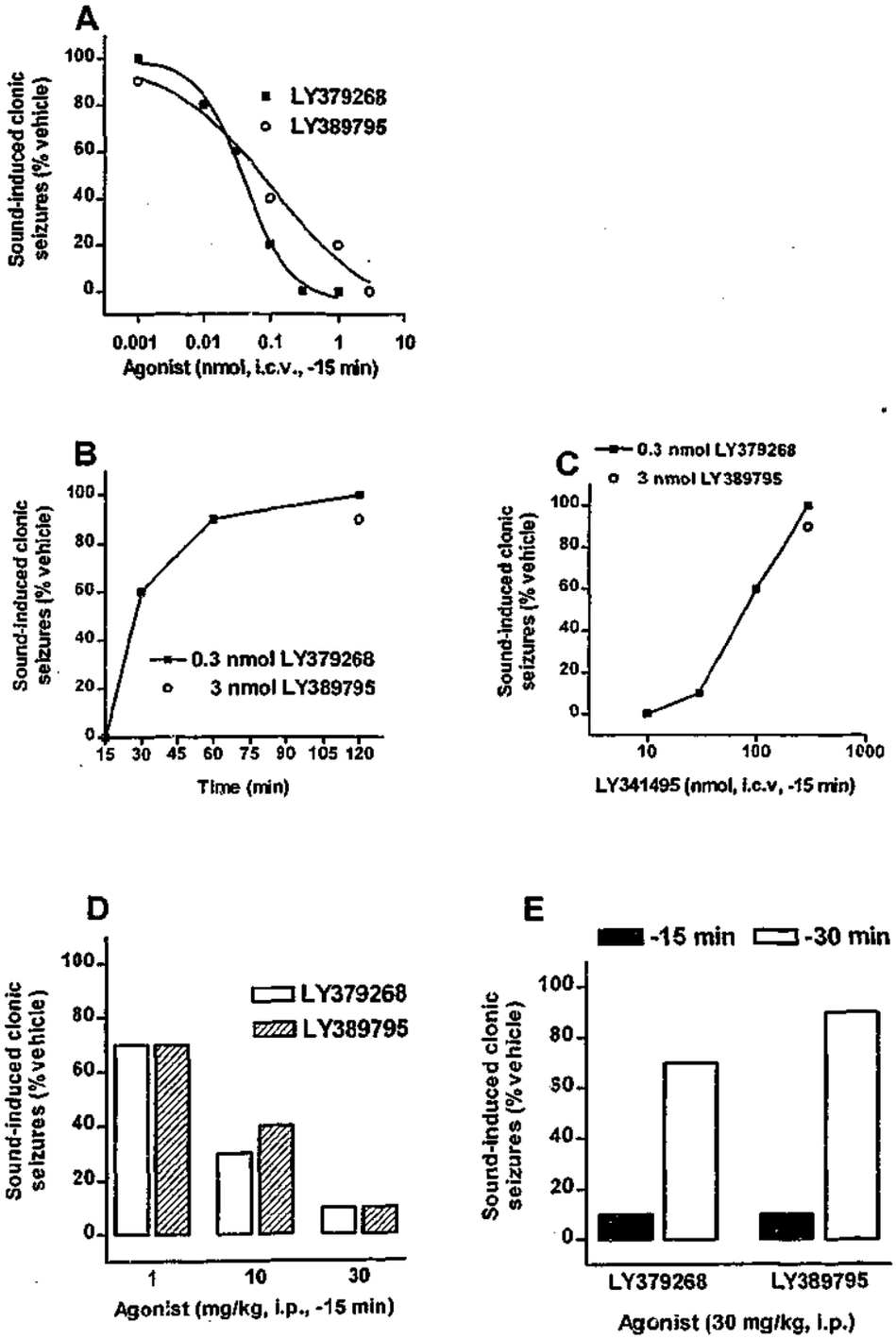
B. Time-dependent loss of anticonvulsant activity.

C. Dose-dependent suppression of agonist inhibition of seizures by the $mGlu_{2/3}$ antagonist LY341495.

D. Dose-dependent inhibition of seizures by the agonists i.p.

E. Loss of anticonvulsant activity of the agonists at 30 min, i.p. $n = 10$.




A
Sound-induced clonic seizures (% vehicle)
LY379268
LY389795
Agonist (nmol, i.c.v., -15 min)
B
Sound-induced clonic seizures (% vehicle)
0.3 nmol LY379268
3 nmol LY389795
Time (min)
C
0.3 nmol LY379268
3 nmol LY389795
Sound-induced clonic seizures (% vehicle)
LY341495 (nmol, i.c.v, -15 min)
D
Sound-induced clonic seizures (% vehicle)
LY379268
LY389795
Agonist (mg/kg, i.p., -15 min)
E
-15 min
-30 min
Sound-induced clonic seizures (% vehicle)
LY379268
LY389795
Agonist (30 mg/kg, i.p.)

following i.c.v. injection of LY379268 was not significantly different from that of vehicle control (P = 0.1903; clonic seizures shown in Figure 4.4B). The $mGlu_{2/3}$ antagonist LY341495 (10–300 nmol, i.c.v.) suppressed the inhibition of sound-induced seizures and score of 0.3 nmol LY379268 in a dose-dependent manner (IC_{50} = 110.0 [9.3–430.7] nmol ($P \leq 0.0052$; Figure 4.4D)). LY341495 (300 nmol) similarly suppressed the inhibition of sound-induced seizures and score by 3 nmol LY389795 (P = 0.0001; Figure 4.4D).

Following i.p. administration, LY379268 and LY389795 (1–30 mg/kg) inhibited sound-induced seizures and score in a dose-dependent manner whereby ED_{50} = 2.9 [0.9–9.6] mg/kg, E_{max} = 10% of vehicle (30 mg/kg) ($P \leq 0.0021$; Figure 4.3D) and ED_{50} = 3.4 [1.0–11.7] mg/kg, E_{max} = 10% of vehicle (30 mg/kg) ($P \leq 0.0433$; Figure 4.3D), respectively. A significant loss of anticonvulsant activity was observed at 30 min compared to 15 min following i.p. administration of LY379268 and LY389795 (30 mg/kg; P = 0.0288 and P = 0.0007; Figure 4.3E). DBA/2 mice demonstrated no proconvulsant behaviour following i.p. injection of the agonists and appeared only slightly sedated following administration of 30 mg/kg.

4.3.2. Rotarod performance in DBA/2 mice

LY379268 and LY389795 produced a dose-dependent impairment of motor performance on the rotarod when DBA/2 mice were injected i.c.v. whereby ED_{50} = 2.2 [6.4–0.7] nmol (LY379268) and ED_{50} = 1.5 [5.4–0.5] nmol (LY389795). The therapeutic indices (rotarod locomotor deficit ED_{50}/anticonvulsant ED_{50}) for the two agonists were 27.5 for LY379268, and 1.8 for LY389795. At 30 mg/kg, LY379268 and LY389795 produced an impairment of motor performance of 45% and 68%, respectively. 2*R*,4*R*-

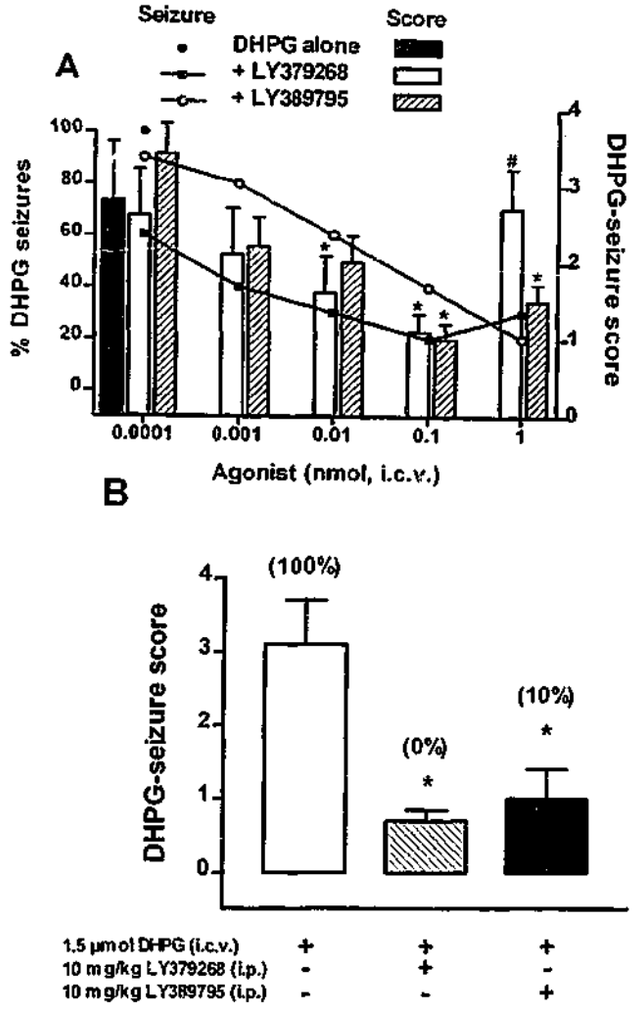
APDC (20 nmol, i.c.v.) produced a rotarod performance that was 100% of that tested prior to injection (n = 9).

4.3.3. Inhibition of DHPG-induced seizures in DBA/2 mice

When co-injected with 1.5 μmol DHPG, 2*R*,4*R*-APDC (10-40 nmol) significantly inhibited DHPG-induced seizure-related behaviour (DHPG seizure score) including hindlimb scratching, mouth and forelimb clonus, rearing and falling, and head and tail extension over a timecourse of 90 min. The significant inhibition of the DHPG seizure score by 10-40 nmol 2*R*,4*R*-APDC ($P \leq 0.0011$) is presented in Figure 4.3D. The inhibition of DHPG seizure score by 20 nmol 2*R*,4*R*-APDC is significantly different from the inhibition produced by 10 ($P = 0.0002$) and 40 nmol ($P = 0.0003$) 2*R*,4*R*-APDC, indicating a reversal of anticonvulsant activity similar to that observed in sound-induced seizures.

LY379268 and LY389795 (0.0001–1 nmol, i.c.v.) produced a dose-dependent inhibition of DHPG-induced seizures and seizure score ($P \leq 0.0216$, 0.01–0.1 nmol LY379268; $P \leq 0.0262$, 0.1–1 nmol LY389795; Figure 4.5). LY379268 inhibited DHPG-induced seizures whereby ED_{50} = 0.3 [0.02–5.0] pmol and E_{max} = 20% of DHPG alone. LY389795 inhibited DHPG-induced seizures whereby ED_{50} = 0.03 [0.05–0.19] nmol and E_{max} = 20% of DHPG alone. An increase in DHPG-induced seizures and seizure score was found at agonist doses of > 1 nmol when compared to doses of 0.01–0.1 nmol. This increase in DHPG-induced seizure score included occasional respiratory arrest approximately 30 min following i.c.v. co-injection of either agonist and DHPG. A slight increase in DHPG-induced seizures and a significant increase in DHPG-induced score following i.c.v. co-injection with 1 nmol LY379268 compared to 0.1 nmol is presented in Figure 4.5 ($P = 0.011$). Both agonists inhibited DHPG-induced seizures and seizure score

Figure 4.5 Inhibition of DHPG-induced seizures.



A. The dose-dependent inhibition of seizures (line graph) and seizure score (bar graph) by the agonists co-injected i.c.v. with 1.5 µmol DHPG.

B. Inhibition of seizures (in parenthesis) and seizure score following i.p. injection of the agonists 50 min after i.c.v. injection of DHPG. Seizure score data is represented as the mean ± S.E.M. of n = 10.

* $P < 0.05$ compared to DHPG alone.

\# $P < 0.05$ compared to 0.1 nmol LY379268: Mann-Whitney U test.

when administered systemically. When administered 50 min following the i.c.v. injection of 1.5 μmol DHPG, 10 mg/kg LY379268 and LY389795 inhibited DHPG-induced seizures to 0% and 10% of vehicle, respectively (Figure 4.5B).

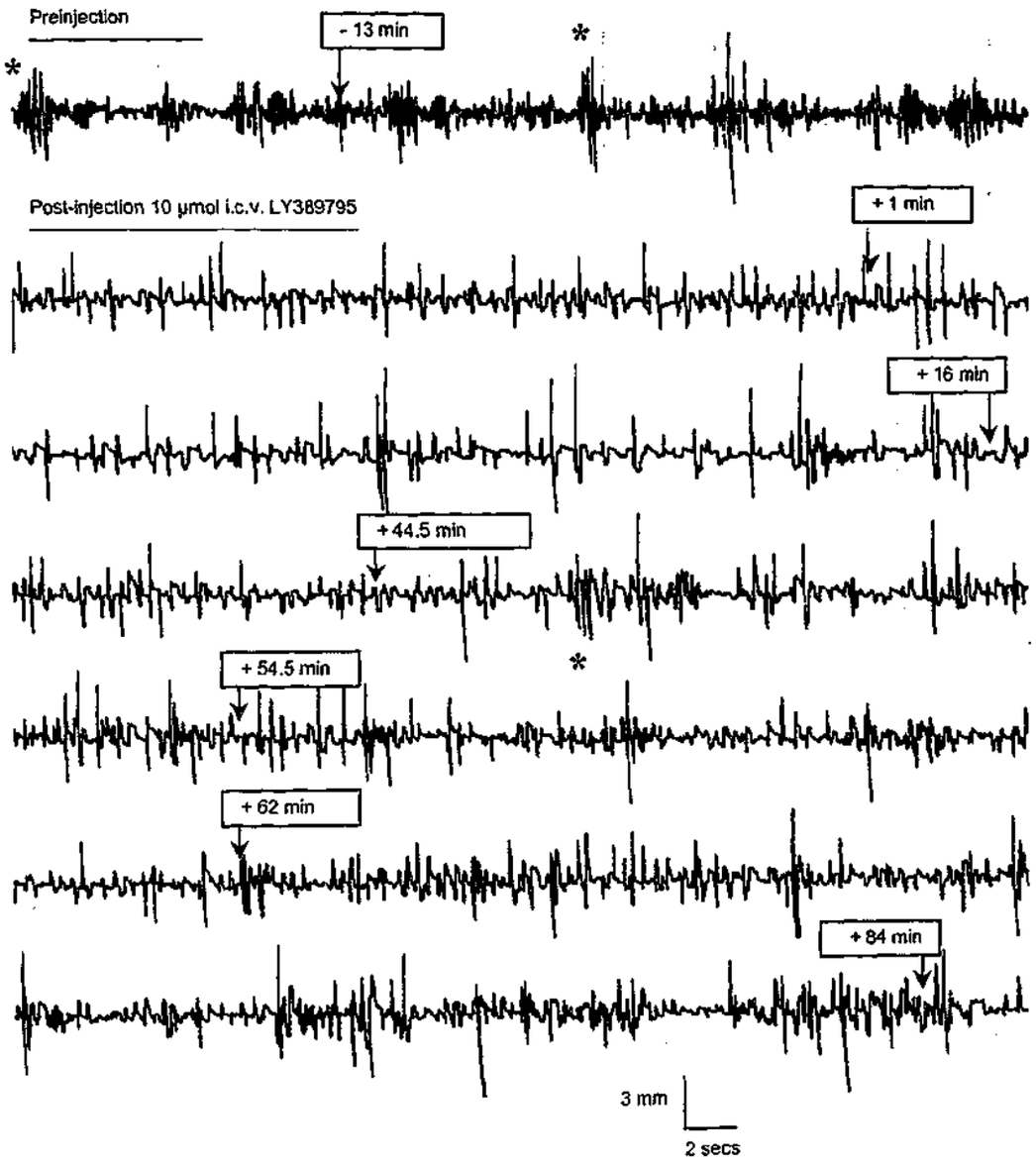
4.3.4. Sound-induced seizures in GEP rats are not inhibited by i.p. administration of the agonists

GEP rats were tested for sound-induced seizures 30 min or 1 h following i.p. administration of LY379268 and LY389795 (0.1–1 mg/kg). The agonists did not inhibit score 9 seizures in GEP rats at either timepoint at any of the doses tested. At doses ≥ 0.1 mg/kg the agonists induced proconvulsant activity following the sound-induced score-9 seizure stimulated at 30 min and 1 h (43% of animals tested). This proconvulsant activity was generally characterised by ataxic hyperlocomotion for approximately 15–20 min following the score-9 seizure and concluded in whole-body clonus: for this reason higher doses were not tested. At both timepoints and across both agonists, the dose of 0.1 mg/kg induced proconvulsant activity following the sound-induced seizure in 6% of animals tested. In the absence of the sound stimulus proconvulsant activity was not observed at any of the doses tested.

4.3.5. Inhibition of spike and wave discharge in lh/lh mice

Intracerebroventricular administration of the LY379268 and LY389795 (1 and 10 nmol) reduced the duration of spike and wave (SWD) discharge in *lh/lh* mice compared to vehicle up to 90 min following injection ($P < 0.05$, LY379268 and LY389795; one-way ANOVA). Figure 4.6 presents a representative EEG trace showing the SWD of absence seizures, before and following LY389795 treatment. The reduction of quantified SWDs by the agonists is presented in Figure 4.7. Administration of 1 and 10 nmol LY379268

Figure 4.6 Representative EEG traces of cortical spike and wave discharge of absence seizures in *lh/lh* mice before and following infusion of 10 µmol LY389795.
* indicates some typical absence seizures.



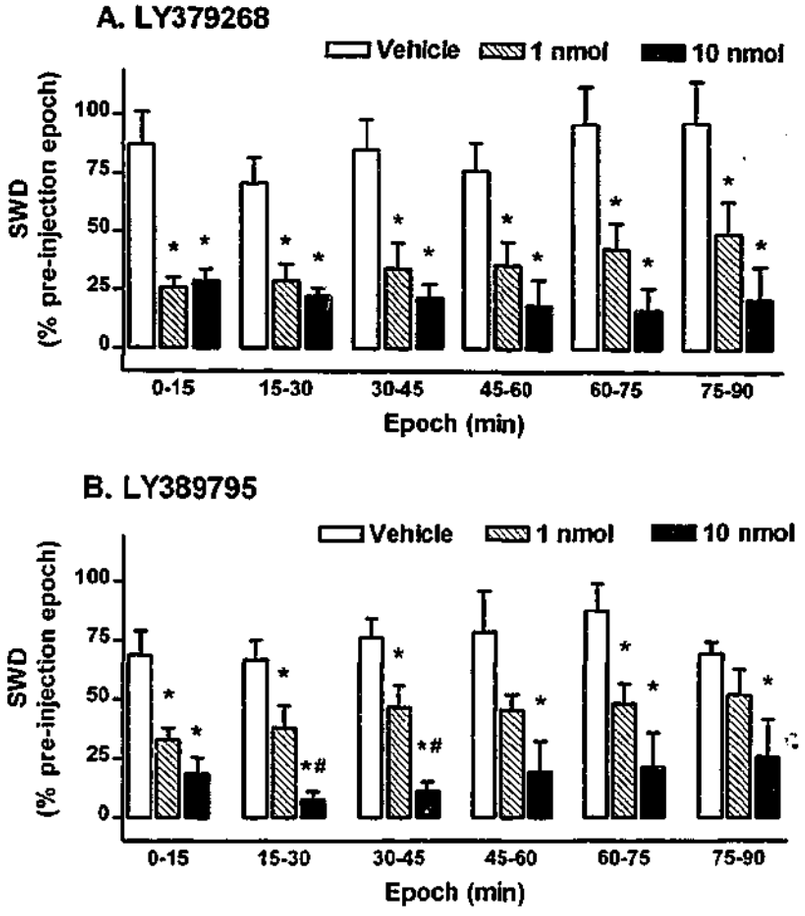


Figure 4.7 Inhibition of spike and wave discharge (SWD) in *Ih/Ih* mice.

Significant inhibition of the duration of SWD by 1 and 10 nmol LY379268 (A) and LY389795 (B), i.c.v. SWD data is represented as the mean ± S.E.M. of $n = 5–6$.

* $P < 0.05$ compared to vehicle, One-way ANOVA.

# $P < 0.05$ compared to 10 nmol LY389795, Student Newman Keuls *post hoc* test.

significantly inhibited SWD up to 90 min following injection. Administration of 1 nmol LY389795 significantly inhibited SWD up to 75 min, while 10 nmol inhibited SWD for up to 90 min. Additionally, 10 nmol LY389795 significantly reduced SWD in *lh/lh* mice compared to 1 nmol from 15–45 min following injection ($P < 0.05$; Newman-Keuls *post hoc*). Proconvulsant activity was not observed following i.c.v. administration of the agonists, some slight sedation occurred in those *lh/lh* mice receiving 10 nmol of either agonist.

4.3.6. Inhibition of electrically-stimulated seizures in amygdala-kindled rats

LY379268 and LY389795 produced a partial inhibition of electrically-induced seizure score in Am-kindled rats at 30 min following i.p. injection of 10 mg/kg ($P \leq 0.0032$; Figure 4.8). Figure 4.9 presents a representative EEG trace showing the SWD of absence seizures, before and following LY389795 treatment. Both agonists significantly decreased the duration of spike and wave discharge (SWD) of the cortices 30 min following injection of 10 mg/kg, i.p. ($P \leq 0.0004$; Figure 4.10). (Due to the severity of electrically-induced seizures in Am-kindled rats, the animals were never repeatedly stimulated within 5 h after receiving the first stimulation). LY379268 similarly inhibited the duration of amygdala SWD under these conditions ($P = 0.0012$), which was mostly absent by 6 h ($P \geq 0.6632$). The inhibition of seizure score was absent when the animals were again stimulated 6 h following injection ($P = 0.3739$, LY379268 and LY389795); which concurred with the loss of the inhibition of SWD duration following LY379268 treatment, but contrasted with the duration of SWD following treatment with LY389795. Following i.p. injection of 10 mg/kg LY389795, a significant inhibition of the duration of cortical and amygdaloid SWD was obtained at 6 h ($P \leq 0.0361$; Figure 4.10). A significant inhibition of electrically-induced seizure score was not achieved with either agonist at 3 mg/kg ($P \geq 0.208$). However, while

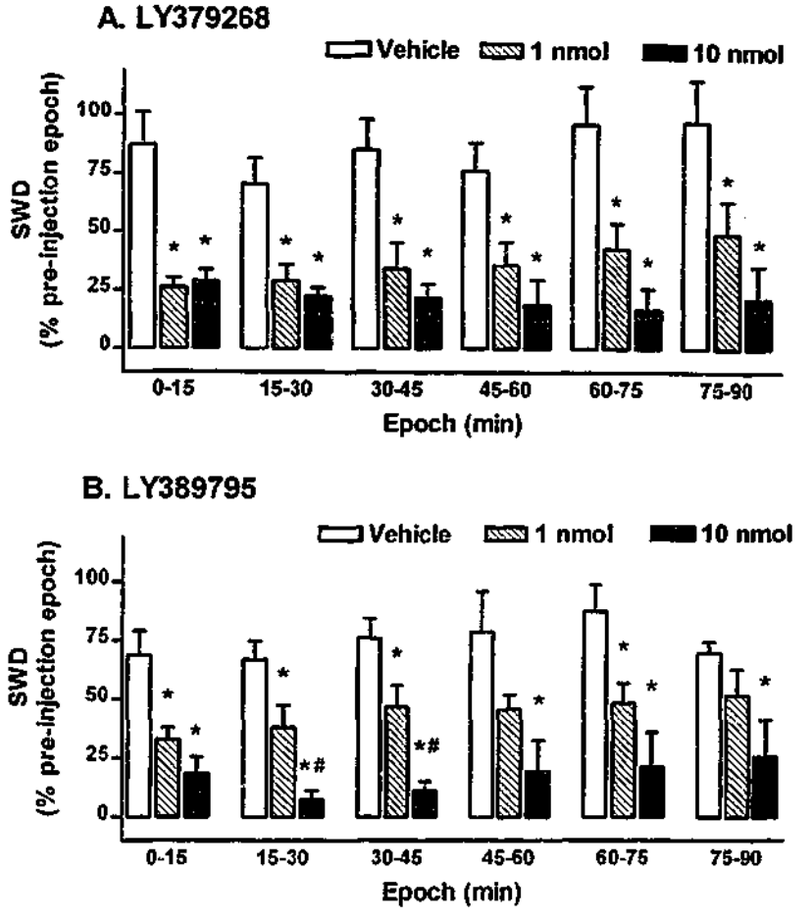


Figure 4.7 Inhibition of spike and wave discharge (SWD) in *lh/lh* mice.

Significant inhibition of the duration of SWD by 1 and 10 nmol LY379268 (A) and LY389795 (B), i.c.v. SWD data is represented as the mean ± S.E.M. of n = 5–6.

* $P < 0.05$ compared to vehicle, One-way ANOVA.

# $P < 0.05$ compared to 10 nmol LY389795, Student Newman Keuls *post hoc* test.

Figure 4.9. Representative EEG traces of electrically-stimulated Am-kindled rats The spike and wave discharge of the amygdala (A & C) and cortices (B & D) 24 h prior to (A & B), and 30 min following (C & D) administration of LY389795 (10 mg/kg, i.p.).

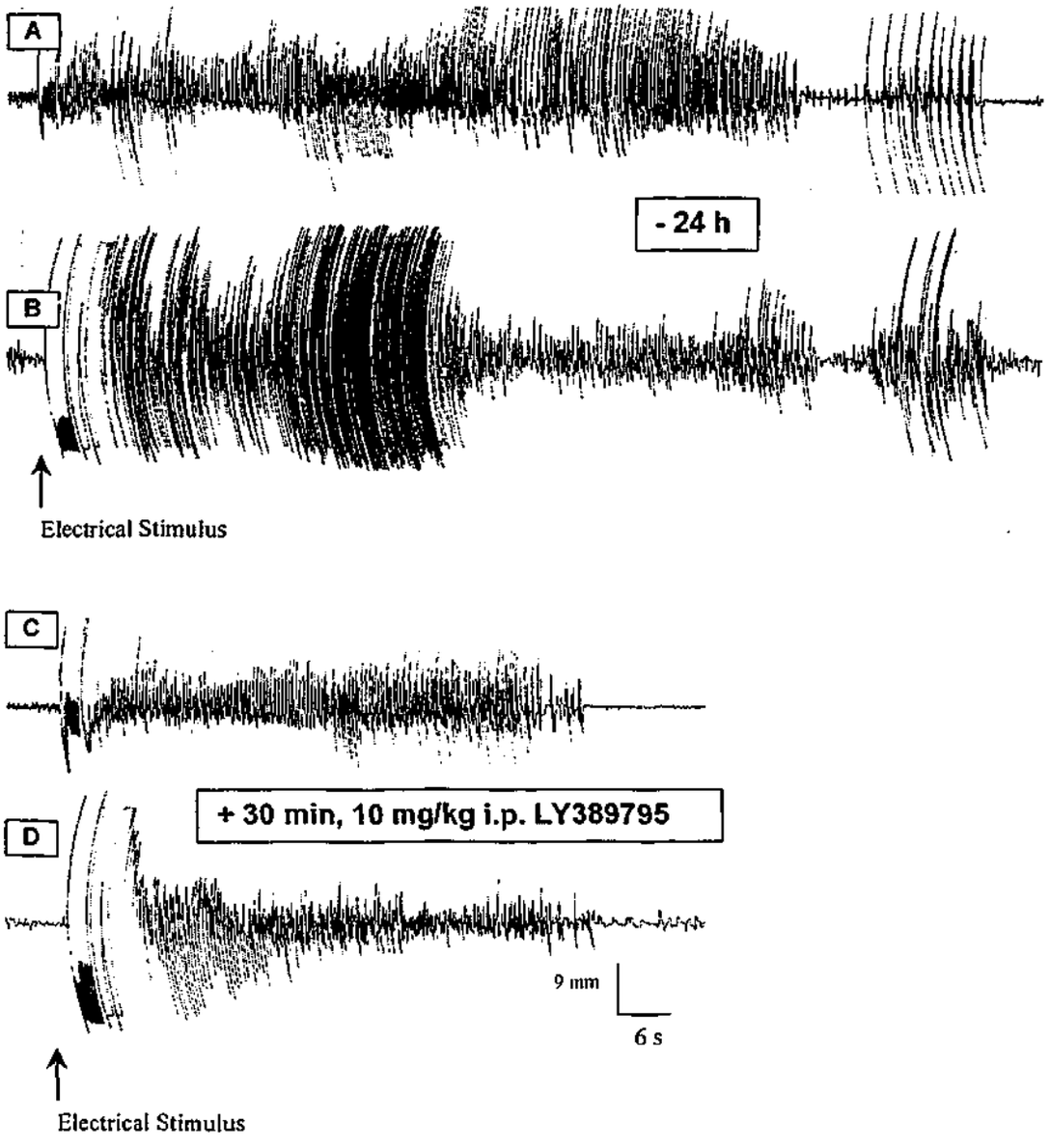
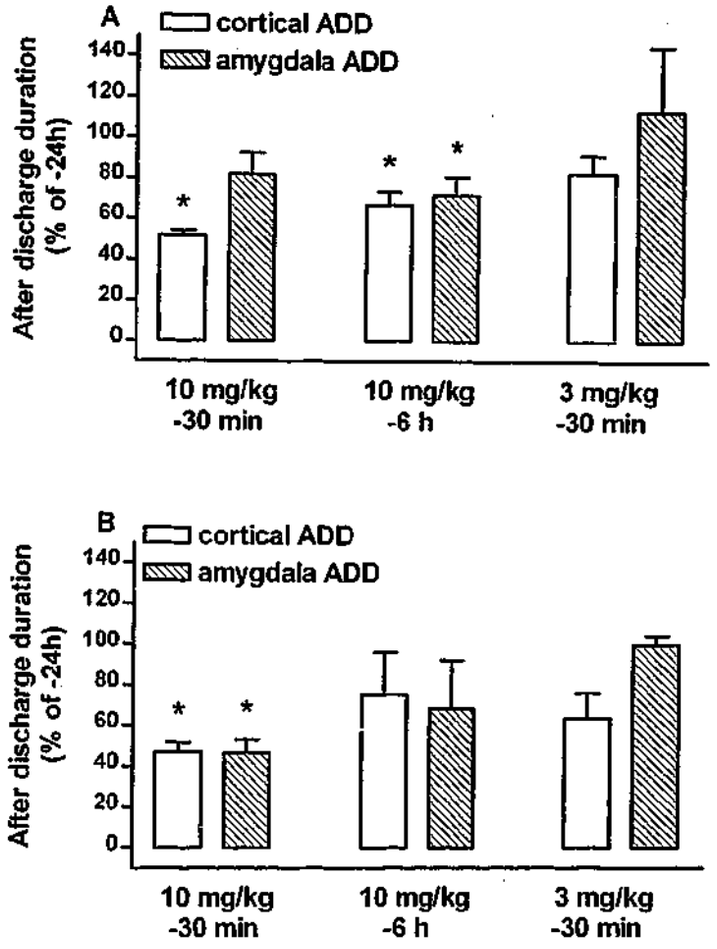


Figure 4.10 Inhibition of the duration of spike and wave discharge (SWD) in amygdala-kindled rats following i.p. administration of LY379268 (A) and LY389795 (B). The duration of SWD is expressed as a percentage of the –24 h control and the treatment group presented as mean ± S.E.M.

*$P < 0.05$, duration of SWD of the treatment compared to the respective –24 h control, paired Student t-test. $n = 5$.



not significant, a partial inhibition of the duration of cortical SWD was observed with either agonist 30 min following this latter dose (Figure 4.10). Proconvulsant activity was observed following i.p. administration of the agonists at 30 mg/kg (n = 2–3). This proconvulsant activity was characterised by head-turning and spontaneous jumping 15–50 min following injection.

4.3.7. Proconvulsant activity induced by i.c.v. administration of 2R,4R-APDC, LY379268 and LY389795 in DBA/2 mice

The proconvulsant profile of the agonists was determined by i.c.v. injection at doses of 0.1–3 μmol in DBA/2 mice for 90 min. LY379268 induced intermittent and spontaneous wild running and hindlimb scratching at 1 and 3 μmol. In contrast, at 0.3–3 μmol 2*R*,4*R*-APDC and LY389795 rapidly induced wild running, hindlimb scratching, clonus, tonus and respiratory arrest. LY389795-induced clonus consisted of head and tail extension, unilateral or bilateral forelimb clonus, and whole-limb clonus (Table 4.1).

4.4. Discussion

This study evaluated the anti-epileptic activity of three novel agonists for group II mGlu receptors in various animal models of epilepsy. The agonists, 2*R*,4*R*-2R,4R-APDC, LY379268 and LY389795 were anti-epileptic in generalised motor seizures in DBA/2 mice, primary generalised non-convulsive seizures in *lh/lh* mice, epileptogenesis induced in rats, and chemoconvulsant seizures in DBA/2 mice involving the recruitment of the limbic system. The agonists were not anti-epileptic the rat model of generalised motor seizures. 2*R*,4*R*-APDC was not used throughout all studies because of the proconvulsant activity it produced in the sound-induced seizures of DBA/2 mice, and also because the more

Table 4.1 Occurrence of $mGlu_{2/3}$ agonist- and antagonist-induced seizure activity in DBA/2 mice

$mGlu_{2/3}$ ligand	µmol (i.c.v.)	Hindlimb Scratching	Mouth/FL Clonus	Clonus	Tonus	R.A.	Score[a]
Vehicle	0	1/6	0/6	0/6	0/6	0/6	0.17 ± 0.17
2*R*,4*R*-APDC	0.1	2/6	0/6	0/6	0/6	0/6	0.33 ± 0.21
	0.3	5/6	2/6	3/6	0/6	0/6	1.50 ± 0.56*
	0.6	6/6	6/6	6/6	1/6	1/6	3.17 ± 0.40*
	1.0	6/6	6/6	6/6	1/6	4/6	3.83 ± 0.31*
LY389795	0.1	6/6	0/6	0/6	0/6	0/6	1.00 ± 0.00*
	1.0	6/6	1/6	6/6	1/6	1/6	3.33 ± 0.42*
	3.0	6/6	6/6	6/6	3/6	4/6	4.83 ± 0.60*
LY379268	3.0	2/6	0/6	0/6	0/6	0/6	0.83 ± 0.40
Vehicle	0	2/10	0/10	0/10	0/10	0/10	0.20 ± 0.13
LY341495	0.1	3/10	0/10	0/10	0/10	0/10	0.30 ± 0.15
	0.3	0/10	3/10	2/10	1/10	1/10	1.20 ± 0.14*
	0.6	3/10	8/10	8/10	7/10	7/10	4.30 ± 0.72*
	1.0	8/10	10/10	10/10	10/10	10/10	6.60 ± 0.16*

[a] Score represents the mean ± S.E.M. of the occurrence of seizure activity (hindlimb scratching, mouth clonus, clonus, tonus and respiratory arrest) per animal in the treatment group. * $P < 0.05$ compared to vehicle (Mann-Whitney U test). FL; forelimb. R.A.; respiratory arrest.

selective, potent and systemically active agonists, LY379268 and LY389795, would suffice to demonstrate the role of group II mGlu receptors in preventing the seizures of these animals.

The $mGlu_{2/3}$ agonists, 2*R*,4*R*-APDC, LY379268 and LY389795 have been shown in the present study to inhibit DHPG-induced seizures, which are understood to recruitment limbic brain regions (Tizzano et al., 1995). These findings concur with those of Monn et al., (1997, 1999) where these agonists and the structurally related LY354740 was shown to inhibit 1*S*,3*R*-ACPD-induced limbic seizures following i.p. injection. In agreement with previous studies showing that LY354740 inhibited chemoconvulsant clonic seizures following i.p. injection (Klodzinska et al., 2000), the present study has also demonstrated the inhibition of clonic seizures induced by sound following i.p. administration of the more selective $mGlu_{2/3}$ agonists LY379268 and LY389795.

In general, LY379268 appeared more potent at inhibiting seizures across all models than LY389795; which is in agreement with their respective potencies for $mGlu_{2/3}$ (Schoepp et al., 1999). When administered i.c.v. this difference in potency was approximately 10-fold, however when administered i.p. the potency of the agonists was relatively similar. Furthermore, LY379268 demonstrated a more favourable therapeutic index than LY389795. The selectivity and potency of the agonists for group III mGlu receptors at higher doses cannot be ruled out from consideration of the differences in activity observed (Schoepp et al., 1999). At these higher doses, LY379268 is more likely to activate group III mGlu receptors than LY389795. This is possibly why at doses of 1–3 μmol i.c.v., LY379268 does not induce clonus, unlike LY389795. The latter agonist not only demonstrates a similar proconvulsant profile at high doses to 2*R*,4*R*-APDC but also shares a similar mGlu receptor agonist profile to 2*R*,4*R*-APDC. In particular, LY389795 and 2*R*,4*R*-APDC have

not been reported to activate group III mGlu receptors at concentrations comparable to LY379268 (Schoepp et al., 1999). LY379268 and LY389795 were approximately 250-fold and 25-fold more potent at inhibiting sound-induced seizures than the $mGlu_{2/3}$ agonist 2*R*,4*R*-APDC respectively, and at least 600-fold more potent at inhibiting DHPG-induced seizures than 2*R*,4*R*-APDC (i.c.v.). Additionally, LY379268 and LY389795 were approximately 20-fold more potent at inhibiting sound-induced seizures when administered systemically than 2*R*,4*R*-APDC.

Sound-induced clonic seizures in DBA/2 mice are produced by activation of auditory structures in brainstem/midbrain regions such as the inferior colliculus and medial geniculate body (Chapman and Meldrum, 1987), while DHPG-induced seizures involve the recruitment of limbic brain regions. Group II mGlu receptor agonists have been reported to inhibit glutamate and aspartate release in numerous brain regions (Cartmell and Schoepp, 2000) and consequently, the anti-epileptic activity seen in the present study most likely results from $mGlu_{2/3}$-mediated inhibition of presynaptic glutamate release from glutamatergic neurones these regions.

LY379268 and LY389795, like 2*R*,4*R*-APDC, were initially reported as a selective agonists for negatively-coupled cAMP-linked mGlu receptors without appreciable activity in group I or III mGlu-expressing cells (Monn et al., 1999; Schoepp et al., 1995). However, 2*R*,4*R*-APDC has since been identified to enhance the phosphoinositide hydrolysis response of the $mGlu_{1/5}$ agonists DHPG and quisqualate in rat hippocampus (Schoepp et al., 1996) – data which may indicate that $mGlu_{2/3}$ has a dual role in the modulation of glutamate release. Since group I mGlu receptors have been identified to induce proconvulsant activity (Tizzano et al., 1995; Dalby and Thomsen, 1996; Chapman et al., 1999; Chapman et al., 2000), it might be expected that $mGlu_{2/3}$ agonists would augment

seizure activity during activation of group I mGlu receptors. For example, in the absence of selective $mGlu_{1/5}$ agonists, activation of $mGlu_{1/5}$-coupled second messenger pathways during sound-induced seizures by endogenous glutamate, which promotes clonus, could be augmented by high doses of $mGlu_{2/3}$ agonists. Evidence to support this hypothesis was observed during evaluation of the anticonvulsant activity of 2*R*,4*R*-APDC, and earlier by Attwell and colleagues (1998b). 2*R*,4*R*-APDC was shown to be anticonvulsant in models of sound and DHPG-induced seizures, however at doses two times that of the anticonvulsant dose, 2*R*,4*R*-APDC was proconvulsant. Similarly, in the present study, LY379268 and LY389795 induced proconvulsant activity in the model of DHPG ($mGlu_{1/5}$ agonist)-induced seizures at higher doses – consistent with the hypothesis for a dual role mediated by $mGlu_{2/3}$. However, the current agonists showed a demonstrable dose-dependent inhibition of DHPG-induced seizure activity, unlike that seen for 2*R*,4*R*-APDC, which may result from their differing potencies for $mGlu_{2/3}$.

Contrasting results found in the DBA/2 mice and the GEP rats is surprising in that previous studies of mGlu receptor related agonists and ionotropic glutamate receptor antagonists have given consistent results in the two seizure models (Meldrum and Chapman, 1999). A possible explanation for these differences could be due to the loss of $mGlu_{2/3}$ expression and function during development (Ross et al., 2000). The GEP rats are developmentally more advanced than the DBA/2 mice and may have lost responsiveness to $mGlu_{2/3}$ agonists at specific sites involved in epileptogenesis. However, focal injection of $mGlu_{2/3}$ agonists into the key epileptogenic regions such as the inferior colliculus (Tang et al., 1997) or the substantia nigra (personal communication: B.S. Meldrum) of GEP rats does inhibit the induction of sound-induced seizures. Whether i.p. administration of LY379268 or LY389795 similarly function in these regions is unclear. LY379268 (intravenous)

induction of glucose utilization has been demonstrated in the inferior colliculus (Lam et al., 1999), but only at doses 30 times higher than that which produced proconvulsant activity in the present study. Furthermore, LY379268 (intravenous)-induced glucose utilization has not been demonstrated in the substantia nigra (Lam et al., 1999); which may explain why an inhibition of sound-induced seizures was not observed following i.p. administration of LY379268 and LY389795 in GEP rats in the present study. Ultimately, whether because of a failure to reach key epileptogenetic regions or due to a developmentally-regulated loss of $mGlu_{2/3}$ sensitivity, when administered systemically, $mGlu_{2/3}$ agonists are not anticonvulsant in the GEP rat model of generalised seizures.

Repeated stimulation of structures within the limbic system (kindling), for example the amygdala, permanently lowers the seizure threshold of that particular region (Meldrum et al., 1999). This process of epileptogenesis is thought to involve enhancement of voltage-sensitive Ca^{2+} conductance, mGlu receptor-mediated phosphoinositide hydrolysis and glutamate release (Meldrum et al., 1999). Consequently, the inhibition of electrically-stimulated seizure activity produced by the agonists in the present study most likely results from the inhibition of glutamate release and voltage-sensitive Ca^{2+} conductance. Conversely, the proconvulsant activity seen with higher doses of the agonists may result from $mGlu_{2/3}$-mediated synergy of $mGlu_{1/5}$ activation. Evidence suggests that this $mGlu_{2/3}$ agonist-induced anticonvulsant and convulsant action may take effect in the amygdala itself (Suzuki et al., 1996; Attwell et al., 1998a). However, intravenous administration of LY379268 failed to induce glucose utilization in the amygdala (Lam et al., 1999). While this does not disclude the amygdala from mediating this dual role, it also indicates that the partial inhibition of electrically-stimulated seizures shown here may result via inhibition of glutamate release in recruited structures of the cortical or subcortical regions. This

hypothesis is supported by the observation that, in general, the inhibition of cortical spike and wave discharge was often greater than that of the amygdala.

LY379268 and LY389795 showed inhibition of cortical and amygdaloid SWD for up to 6 h following i.p. injection. Variability in the responses of kindled-rats following i.p. injection of LY379268 at 6 h was most likely responsible for the difference between the two agonists under these conditions. A greater latency in the inhibition of amygdaloid SWD was observed following i.p. administration of LY389795 than following administration of LY379268. Similarly, a delay in the inhibition of electrically-stimulated seizure score has been described following intra-amygdaloid injection of 1*S*,3*R*-ACPD into Am-kindled rats previously (Suzuki et al., 1996).

The present study is the first to show that $mGlu_{2/3}$ agonists inhibit the duration of SWD of *lh/lh* mice. Single gene mutations that comprise the β4-subunit of voltage-sensitive Ca^{2+} channels underlie the spontaneous discharges of the absence, non-convulsive seizures of *lh/lh* mice. Beta subunits have been shown to attenuate G protein-coupled inhibition of Ca^{2+} channels (Campbell et al., 1995), however recent evidence indicates that an increase in β3-subunit expression compensates for the 'defective' β4 subunits of *lh/lh* mice, except in some areas, including the thalamus and neocortex (Tanaka et al., 1995; Burgess et al., 1999; Lin et al., 1999). Hence, it is possible that $mGlu_{2/3}$ agonists, acting via G protein-mediated inhibition of Ca^{2+} channels in those β4-subunit 'defective' regions, reduce the duration of SWD in *lh/lh* mice. However, further elucidation of the pathological implications behind the mutation to the β4-subunit gene is required to fully understand the mechanisms behind the observations of the present study.

The pharmacological profile of the $mGlu_{2/3}$ antagonist LY341495 is dissimilar to that of the selective agonists when given by i.c.v. injection. Despite a difference in action,

anticonvulsant versus proconvulsant activity, none-the-less LY341495 is less potent a proconvulsant as the agonists are anticonvulsant. In fact both 2*R*,4*R*-APDC and LY389795 are almost equally potent as proconvulsants as the antagonist. This observation suggests that while $mGlu_{2/3}$ are important sites in anticonvulsant behaviour they are not responsible for proconvulsant behaviour. They same dynamics does not apply to AMPA receptors for example; with these iGlu receptors, agonists and antagonists are equally potent inducers of proconvulsant or anticonvulsant activity respectively (Chapman et al., 1991). The proconvulsant activity of $mGlu_{2/3}$ agonists and possibly antagonists appears due to their grossly non-selective and toxic actions. The proconvulsant activity reported here merely establishes the upper limits of a safe anticonvulsant dose range. The exception is 2*R*,4*R*-APDC, where proconvulsant activity was recorded at doses less than 100 nmol i.c.v. in the sound and DHPG seizure models. However this proconvulsant activity did not immediately manifest itself in a similar way to that of LY389795 or LY341495 at these lower doses, instead similar high doses were required to elicit the proconvulsant behaviour of wild running, mouth clonus, clonus and tonus. If the proconvulsant activity of 2*R*,4*R*-APDC was produced by activation of $mGlu_{2/3}$ it would be expected that LY379268 and LY389795, with higher selectivity for $mGlu_{2/3}$, would be more potent at inducing proconvulsant behaviour. However, this was not observed; instead no dose of LY379268 in DBA/2 mice induced clonus. 2*R*,4*R*-APDC is unique in this low-dose proconvulsant effect, but why this is the case is unknown at present.

This study has shown that group II mGlu receptors play a role in the amelioration of seizures in animals which share a similar etiology to those seizures found in humans. Furthermore, systemic administration of LY379268 and LY389795 in particular inhibit these seizures and consequently demonstrate potential as potent anti-epileptic drugs.

CHAPTER 5

GENERAL DISCUSSION

Studies within the present thesis have examined the function of group II metabotropic glutamate receptors using a range of pharmacological tools under normal and pathological conditions, including toxicity *in vitro* and epilepsy *in vivo*.

The role of Glu and the glutamatergic neurotransmission system has been described in detail in Chapter 1. From this introduction it is clear that disruption of Glu homeostasis is known to cause neuronal injury and/or convulsive seizures, and is involved in the neuropathology of many neurological disorders including Alzheimer's disease, amyotrophic lateral sclerosis, ischaemic stroke and epilepsy.

Since group II mGlu receptors are located on the presynaptic terminal and inhibit glutamatergic neurotransmission (Anwyl, 1999; Cartmell and Schoepp, 2000), these receptors represent a potential target for the amelioration of neuronal injury as a result of excitotoxicity. In Chapter 2 the hypothesis that agonists, acting at presynaptic group II mGlu receptors could, via inhibition of Glu release prevent the exacerbation of toxicity induced by various insults in cortical, striatal and cerebellar granular pure neuronal cultures was investigated. The presence of functional receptors in these preparations was confirmed by 2*R*,4*R*-APDC-induced inhibition of forskolin-stimulated production of cAMP. At different days of development *in vitro* a graded pattern of injury to cortical and striatal cultures was achieved through the free radical generator hydrogen peroxide, the non-selective protein kinase inhibitor staurosporine, and the iGlu receptor agonists NMDA, AMPA and KA. Cultures were also exposed to insults over a range of periods to induce varying degrees of injury, due to apoptosis or necrosis, and to reflect some of the cell death seen in neurodegenerative conditions. Granule cells were similarly exposed to low K^+ which is known to induce apoptosis. Some of these injury conditions resembled those used by

previous investigators in cultures of a more mixed cell phenotype whereby group II mGlu receptor agonists had been shown to be neuroprotective (Bruno et al., 1994; Bruno et al., 1997; Bruno et al., 1998a). Treatment of injured cultures with group II mGlu receptor agonists 2*R*,4*R*-APDC, L-CCG-I, DCG-IV and NAAG failed to attenuate the toxicity induced by any of the above insults, under any of the various conditions employed. These findings contrasted to those of Bruno and colleagues in mixed cultures. During the course of this study, Kingston et al., (1999) published findings using similar toxic regimes to those employed here, and found that the third generation group II mGlu receptor agonists LY354740, LY379268 and LY389795 induced neuroprotection that was likely mediated by astrocytes in culture. When the results of these studies are taken with those by Nicoletti and colleagues (Bruno et al., 1998a), and Kingston et al., (1999) together they conclusively show that group II mGlu receptor-mediated neuroprotection *in vitro* can only be achieved by activation of receptors on astrocytes. Furthermore, given the nature of mGlu receptor expression throughout the CNS and *in vitro* (Shigemoto and Mizuno, 2000; Janssens and Lesage, 2001), this neuroprotective effect was most likely mediated by the $mGlu_3$ subtype localised to astrocytes. In 2001, D'Onofrio et al. published their investigation into the signalling mechanisms which revealed a process whereby astrocytes were understood to release TGF-β following $mGlu_3$ activation – a trophic factor responsible for group II mGlu receptor-induced neuroprotection. In brief, TGF-β induction was demonstrated *in vitro* via activation of the MAPK and PI-3-K pathways with similar evidence obtained from *in vivo* experiments.

Despite evidence for the important role of astrocytes that was emerging at that time (as discussed above), signal transduction mechanisms of group II mGlu receptors remained a matter of some controversy, particularly in astrocytes. Therefore, a focused group of

experiments were designed to gain new insights into group II mGlu receptor function in astrocytes by examining inter-relationships of intracellular signalling, particularly in light of our own earlier observation on the sensitivity of $mGlu_{2/3}$-cAMP signalling to extracellular calcium. Chapter 3 demonstrates that under physiological Ca^{2+} and adenylate cyclase stimulation there was a rapid elevation of $[Ca^{2+}]_i$ and an elevation of cAMP production is achieved in astrocytes following group II mGlu receptor stimulation. This observation had been noted earlier in slice preparations (Schoepp et al., 1996a,b), however the interpretations surrounding the basis of this elevation were incomplete and at times contradictory. Furthermore, the data from initial experiments demonstrated that this elevation was not found in neurones and was specific to astrocytes, thereby showing that different cell types possessed different group II mGlu receptor signalling "infrastructure". This elevation in cAMP was subsequently shown to be sensitive to inhibitors of PLC and CaMKII, and the breakdown of endogenous adenosine by adenosine deaminase. While the PLC/IP3 pathway was interpreted to be downstream of group II mGlu receptor activation and precede adenosine release, the role of CaMKII appeared to relate to the adenosine positive feed back on cAMP production. In Chapter 3 also, roles for adenosine in the regulation of glial function and protection of neurones under insult were highlighted. However, further work is needed before it can be concluded that the group II mGlu receptor-mediated release of adenosine in the present study would have a neuroprotective effect *in vitro*. For example, further experiments examining the temporal patterns of involvement of TGF-β and adenosine represent a logical conclusion to the present work. Figure 5.1 outlines some of the key pathways involved in group II mGlu receptor-mediated neuroprotection incorporating possible presynaptic components and what is also now known about the role of astrocytes.

Figure 5.1 Putative neuroprotective pathways induced by activation of group II metabotropic glutamate receptors.

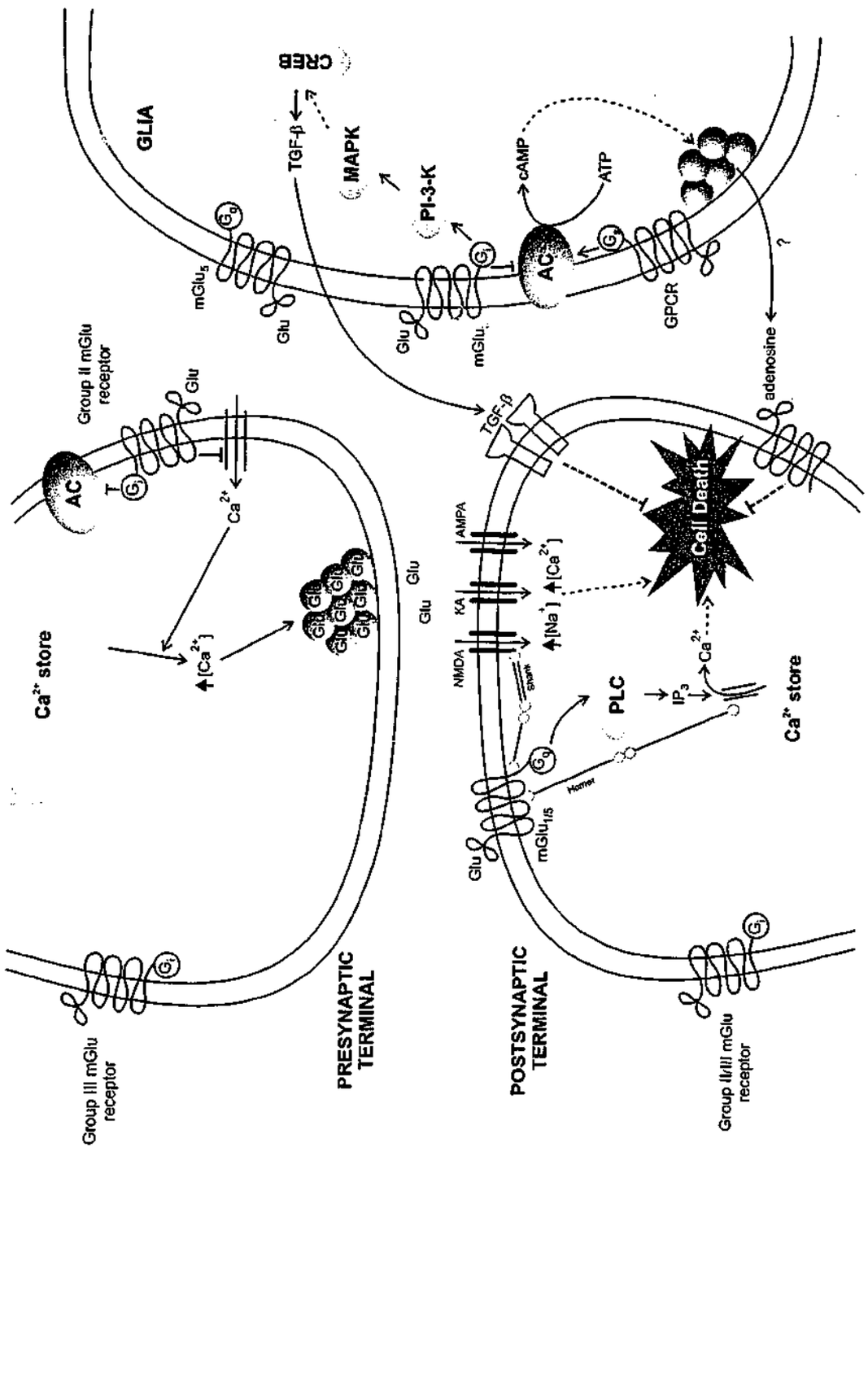
Three neuroprotective pathways postulated in the figure include (1) inhibition of Glu release from activation of presynaptic group II mGlu receptors, (2) induction of trophic factor release from glia (e.g. TGF-β), and (3) induction of adenosine release. Each of these pathways acts to prevent excitotoxicity (cell death) as a result of ion influx in the postsynaptic terminal.

Arrows indicate activation. Lines with T shaped end indicate inhibition. T shaped lines in red highlight the putative neuroprotective pathways.

ctivation of

) inhibition of
(2) induction
of adenosine
l death) as a

on. T shaped



Finally, the ability of group II mGlu receptors to modulate excessive glutamatergic neurotransmission was investigated in animal models of generalised motor seizures, primary generalised non-convulsive seizures, epileptogenesis and convulsions of the limbic system. As a result of these experiments LY379268 and LY389795 were found to be systemically active and potent anticonvulsants, and clearly, these two third-generation $mGlu_{2/3}$ agonists possess anticonvulsant potencies comparable to those of current clinical AEDs (Table 5.1.). Interestingly, unlike LY379268 and LY389795, 2*R*,4*R*-APDC produced marked anti- and proconvulsant activity. The reason for these contrasting results is not entirely clear since their selectivity for group II mGlu receptors is quite similar. 2*R*,4*R*-APDC may produce proconvulsant activity through unique, non-selective actions at other targets of neurotransmission recruited during seizures. Furthermore, this study broadened our understanding of the anticonvulsant potential of group II mGlu receptors, which contrasted to that of some group III mGlu receptors by producing a transient rather than prolonged anticonvulsant effect (Tang et al., 1997).

Other work performed during my doctoral candidature showed that activation of group III mGlu receptors and antagonism of AMPA receptors (using the 3,4-dicarboxyphenylglycines; Thomas et al., 2001) could produce a greater-than-additive anticonvulsant effect than when either of these receptors were targeted alone (Moldrich et al., 2001; Appendix V). In theory, activation of group II mGlu receptors and antagonism of iGlu receptors could produce similar effects to those seen with the 3,4-dicarboxyphenylglycines. More importantly, this study serves to show that targeting multiple sites of the glutamatergic transmission system may produce greater therapeutic benefits than when only one target is addressed, particularly with respect to those conditions which are polygenic or polymorphic. Combination therapy of multiple mGlu and iGlu receptor ligands

Table 5.1 Comparison of the inhibition of sound-induced clonic seizures in DBA/2 mice of conventional anti-epileptic drugs and mGlu receptor ligands. Drugs are listed in decreasing order of potency according to ED_{50} values with 95% confidence intervals.

Drug	Clinical Use	ED_{50} (mg/kg, i.p.)
Diazepam	Partial & generalised seizures	0.28 [0.20–0.39][2]
Phenytoin	Generalised convulsive & partial seizures	2.5 [1.8–3.5][2]
LY379268	n.a.	2.9 [0.9–9.6][6]
Phenobarbital	Partial & generalised seizures	3.4 [2.3–5.0][2]
LY389795	n.a.	3.4 [1.0–11.7][6]
Lamotrigine	Generalised seizures & Lennox-Gastaut syndrome	3.5 [2.4–5.1][2]
Carbamazepine	Generalised convulsive & partial seizures	4.4 [3.6–5.4][2]
Levetiracetam	Adjunct for partial seizures	8.6 [6.2–11.2][1]
Topiramate	Adjunct for partial/generalised seizures	16.2 [11.3–23.1][7]
MPEP	n.a.	18 [10–32][3]
SIB 1893	n.a.	27 [17–44][3]
Sodium Valproate	Partial & generalised seizures	43 [33–56][2]
2*R*,4*R*-APDC	n.a.	75 [25–116][4]
(*RS*)-3,4-DCPG	n.a.	86 [74–101][5]

n.a.: not applicable

[1]Gower et al., 1992 [2]De Sarro et al., 1996 [3]Chapman et al., 2000 [4]Chapter 4 [5]Moldrich et al., 2001 [6]Chapter 4 [7]De Sarro et al., 2000.

with or without current clinical AEDs to achieve a more potent and more broadly-effective anti-epileptic strategy may also serve as an extension to the experiments described in this thesis. In addition to this anti-epileptic strategy, $mGlu_3$ specific agonists may be included to prevent the neuronal loss associated with seizures. The "philosophy" of this glutamatergic, and multi-target approach extends beyond that of epilepsy, and may also apply to stroke, ALS and possibly Alzheimer's disease.

Apart from investigating the temporal pattern of TGF-β and adenosine release from astrocytes (as described earlier), other extensions of this study may include:

- investigating cAMP signalling and adenosine release in mixed, neuronal-glial cultures and organotypic slice cultures,
- using β-adrenoceptor agonists and $mGlu_{2/3}$ agonists to induce adenosine release, and subsequently evaluating potential neuroprotective outcomes,
- using β-adrenoceptor agonists and $mGlu_{2/3}$ agonists to induce a sustained increase in cAMP, which might then be expected to upregulate translation and expression of GluTs in astrocytes (Eng et al., 1997),
- targeting group II mGlu receptor- and astrocyte-mediated neuroprotection in animal models of ischaemic stroke, and parkinsonsim.

In conclusion, potent and selective group II mGlu receptor agonists possess neuroprotective activity *in vitro* and *in vivo* via stimulation of astrocytes, which involves the release of trophic factors and possibly adenosine. The same agonists are also effective at inhibiting seizures in animal models of human epilepsy and demonstrate a potency comparable to clinically effective anti-epileptic drugs. Therefore, such group II mGlu

receptor agonists may prove useful in the treatment of human neurological disorders that involve neurodegeneration due to injury and/or epilepsy.

APPENDICES

Appendix I: Composition of Neurobasal Medium

Brewer, 1995

COMPONENT	mg/L	COMPONENT	mg/L
L-alanine	2	$CaCl_2$.anhydrous	200
L-arginine.HCl	84	$Fe(NO_3)_3.9H_2O$	0.1
L-asparagine.H_2O	0.83	KCl	400
L-cysteine	1.21	$MgCl_2$	77.3
L-glutamine	73.5	NaCl	3000
gylcine	30	$NaHCO_3$	2200
histidine.HCl.H_2O	42	$NaH_2PO_4.H_2O$	125
L-isoleucine	105	D-Ca pantothenate	4
L-leucine	105	folic acid	4
L-lysine.HCl	146	i-inositol	4
L-methionine	30	niacinamide	7.2
L-phenylalanine	66	pyridoxal.HCl	4
L-proline	7.76	riboflavin	4
L-serine	42	thiamine.HCl	0.4
L-threonine	95	vitamin B12	0.34
L-tryptophan	16	D-glucose	4500
L-tyrosine	72	Phenol red	8.1
L-valine	94	HEPES	2600
		sodium pyruvate	25

Appendix II: Composition of B27 components

COMPONENT	CONCENTRATION (μg/ml)
BSA	2500
Biotin	0.1
L-carnitine	2
catalase	2.5
corticosterone	0.02
ethanolamine	1
D(+)-galactose	15
glutathione	1
insulin	4
linoleic acid	1
linolenic acid	1
progesterone	0.063
putrescine	16.1
retinyl acetate	0.1
selenium	0.01
superoxide dismutase	2.5
apo-transferrin	5
triiodothronine	0.02
DL-α-tocopherol	1
DL-α-tocopherol acetate	1

Appendix III:

Composition of N2 Media

COMPONENT	CONCENTRATION (µg/ml)
Insulin (Bovine)	500
Human Transferrin	10000
Progesterone	0.63
Putrescine	1611
Selenite	0.52

Composition of HBSS

COMPONENT	CONCENTRATION (mM)
KCl	5
KH_2PO_4	0.3
NaCl	138
Na_2HPO_4	0.3
D-Glucose	5.6
Phenol Red	0.03

APPENDIX IV: MATERIALS

Chapter 2

Group II mGlu receptor agonists, NMDA, (*S*)-AMPA and KA were purchased from Tocris Cookson (Bristol, UK). LY307452 was a gift from D. Schoepp (Eli Lilly, IN, USA). H_2O_2 and staurosporine were obtained from Sigma (Sydney, Australia). Neurobasal medium, DMEM, B27 and N2 supplements, penicillin/streptomycin, aphidicolin, HBSS, HEPES and L-glutamine were obtained from Gibco-BRL Life Technologies (Melbourne, Australia). Other cell culture components, IBMX, forskolin, MTT and RPMI 1640 medium were purchased from Sigma. MAP-2, NGS and secondary antibodies were acquired from Silenus (Melbourne, Australia), while anti-GFAP was acquired from Incstar (Stillwater, MN, USA). DAB and the Stable Peroxide Substrate were obtained from Pierce (Rockford, IL, USA). The Biotrak [^{3}H]cAMP assay kit was purchased from Amersham (Little Chalfont, UK).

Chapter 3

Metabotropic Glu receptor agonists, *R*,*S*-3,5-dihydroxyphenylglycine (DHPG), 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylic acid (2*R*,4*R*-APDC), and antagonists, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), (αS)-α-amino-α-[(1*S*,2*S*)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY341495) were purchased from Tocris (Bristol, UK) with the exception of (–)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) which was a gift from D.D. Schoepp (Eli Lilly, IN, USA). 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) was also purchased from Tocris. Neurobasal medium, DMEM, B27 supplement, penicillin/streptomycin, HBSS, HEPES, L-glutamine and pertussis toxin were obtained from Invitrogen (Melbourne,

Australia). Other cell culture components, IBMX, forskolin, furosemide, adenosine deaminase, NECA, DMSO, U73122, H89 and the ester of (S)-isoquinolinesulphonic acid (KN-62) were purchased from Sigma (Sydney, Australia). Nifedipine and nimodipine were from RBI (Natick, MA, USA). The Biotrak [^{3}H]cAMP assay kit was purchased from Amersham (Little Chalfont, UK). Pluronic F-127, Fluo-3/AM and the calcium calibration kit were obtained from Molecular Probes (Eugene, OR, USA).

Chapter 4

The selective $mGlu_{2/3}$ agonists (–)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268, M_w = 187.15) and (–)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY389795, M_w = 203.22) were generous gifts from Eli Lilly & Co. 2R,4R-APDC, the $mGlu_{2/3}$ antagonist (α*S*)-α-amino-[(1*S*,2*S*)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY341495) and the $mGlu_{1/5}$ agonist (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG) were purchased from Tocris Cookson Ltd. (Bristol, UK).

APPENDIX V: MOLDRICH ET AL., 2001

DCPG AND EPILEPSY PAPER

Pergamon

Neuropharmacology 40 (2001) 732–735

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Rapid communication

Anticonvulsant activity of 3,4-dicarboxyphenylglycines in DBA/2 mice

Randal X. Moldrich [a], Philip M. Beart [a], David E. Jane [b], Astrid G. Chapman [c], Brian S. Meldrum [c,*]

[a] *Department of Pharmacology, Monash University, Melbourne, Victoria, 3800, Australia*
[b] *Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK*
[c] *Department of Neurology, Institute of Psychiatry, Kings College, London, De Crespigny Park, Denmark Hill, London SE5 8AF, UK*

Received 10 October 2000; received in revised form 4 December 2000; accepted 8 December 2000

Abstract

The 3,4-dicarboxyphenylglycines (3,4-DCPG) inhibit sound-induced seizures in DBA/2 mice with the racemate being notably mor nt than either isomer (ED_{50} (nmol, i.c.v.)): (RS)-3,4-DCPG (0.004; 86 mg/kg, i.p >>the $mGlu_8$ agonist (S)-3,4-DCPG (0.1 >the AMPA antagonist (R)-3,4-DCPG (0.38). A potentiation of anticonvulsant activity between AMPA and $mGlu_8$ receptors was confirmed by combining (R)-3,4-DCPG with the $mGlu_8$ ag RS)-4-phosphonophenylglycine. This potentiating mechanism provides a novel strategy for the treatment of epileptic seizures © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Anticonvulsant; 3,4-dicarboxyphenylglycine (3,4-DCPG); (RS)-4-phosphonophenylglycine (RS)-PPG; DBA/2 mice; Epilepsy; mGluRs

The 3,4-dicarboxyphenylglycines (3,4-DCPG) represent a novel group of pharmacological tools which allow the investigation of the role of glutamate receptors in physiological and pathological conditions. The R- isomer of 3,4-DCPG has been identified as an AMPA receptor antagonist with weak activity at NMDA receptors and little, or no, activity at kainate receptors (Thomas et al., 1997). More recently, the S- isomer has been tested on cloned human metabotropic glutamate receptor (mGlu) subtypes and was identified as a selective and full $mGlu_8$ agonist with weak activity at other mGlu receptor subtypes (Thomas et al., 2001). (S)-3,4-DCPG is at least 100-fold more potent at $mGlu_8$ than other group III mGlu recept h the following order of potency mGl >>mGl >mGl >>$mGlu_7$. Both (RS)- and (S)-3,4-DCPG display weak or no antagonism of NMDA or kainate receptors. A similar functional profile of the compounds has been demonstrated in neonatal rat spinal cord (Thomas et al., 1997, 2001).

Group III mGlu and AMPA receptors have been identified as targets for the suppression of epileptic seizures due to their ability to modulate glutamatergic neurotransmission (Bleakman and Lodge, 1998; Cartmell and Schoepp, 2000). Previous studies have identified group III mGlu receptor agonists as potent inhibitors of sound-induced clonic-tonic seizures in mice and rats following intracerebral injection (Ghauri et al., 1996; Chapman et al., 1999). However, these agonists either induce proconvulsant activity at higher doses or are not anticonvulsant when administered systemically. Alternatively, AMPA receptor antagonists are potent inhibitors of seizures induced in a variety of animal models (Chapman et al., 1991; Meldrum et al., 1992). The present study evaluated the anticonvulsant activity of the (R)-3,4-DCPG, (S)-3,4-DCPG and (RS)-3,4-DCPG against sound-induced clonic-tonic seizures induced in mice and investigated the mechanism behind the potent anticonvulsant activity of (RS)-3,4-DCPG.

All regulated procedures performed in this study were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. Dilute brown agouti (DBA/2) mice (male and female, age 21–28 days, 7–15 g weight; Institute of Psychiatry colony and Harlan and Olac, Bichester, UK) undergo a well-characterised seizure response when exposed to a loud stimulus. Drug or

* Corresponding author. Tel.: +44-207-8480398; fax: +44-207-8480689.
E-mail address: brian.meldrum@kcl.ac.uk (B.S. Meldrum).

0028-3908/01/$ - see front matte
PII: S0028-3908(01)00002-8

vehicle were a[illegible]istered intracerebroventricularly (i.[illegible] under light fluothane anaesthesia in a volume of 1(µl. (RS)-3,4-DCPG was also [illegible]istered intraperitoneally (i.p.) in a volume of 10(µl/10 g body weight. Sound-induced seizures were induced 15–60 min following administration of drug or vehicle as previously described (Chapman et al., 2000). Sound-induced sei[illegible]re activity was scored 0–4 (wild running, clonus, tonus and resp[illegible]y arrest each received a score of 1). The mea ±SEM score per animal within a treatment group was calculated and statistical differences between scores determined by the Mann–Whitney U test. A rotarod test was used to determine drug-induced motor-impairment at 15 min following i.c.v. administration of the compounds as previously described (Chapman et al., 2000). The compounds were synthesised as previously described (Thomas et al., 1997, a fuller account is in preparation) and dissolved in phosphate buffered saline (PBS) for i.c.v. injection where pH 4.0. PBS (pH 4.0) was prepared using HCl and served as the vehicle. (RS)-3,4-DCPG was dissolved in water and adjusted to pH 7.0–7.7 with NaOH for i.p. injection. (RS)-4-phosphonophenylglycine ((RS)-PPG; soluble in PBS) was obtained from Tocris Cookson (Bristol, UK).

Each of the 3,4-DCPG compounds produced a dose-dependent inhibition of sound-induced clonic seizures in DBA/2 mice [illegible] the following ED_{50} values [upper-lower 95% co fidence limits] (nmol, i.c.v.): (RS)-3,4-DCPG (0.0[illegible]0031–0.052[illegible] >>(S)-3,4-DCPG (0.11 [0.054–2.28[illegible] >(R)-3,4-DCPG (0.38 [0.13–1.13]) [Fig. 1(a)]. Sim[illegible], each of the 3,4-DCPG compounds (i.c.v.) sig[illegible]ficantly inhibited the sound-induced seizure score a[illegible]he following doses: 1–10 nmol (R)-DCPG [illegible]≤0.0005), 0.01–10 nmol (S)-3,4-[illegible]≤0.0433), 0.001–10 nmol (RS)-3,4-DCPG [illegible]≤0.0089). The duration of the inhibition of sound-induced seizures was tested at those doses of the compounds which produced 100% inhibition of clonic seizures (3 nmol (R)-3,4-DCPG, 10 nmol (S)-3,4-DCPG and 1 nmol (RS)-3,4-DCPG). Each of the compounds demonstrated a similar transient inhibition of sound-induced seizures such that at 60 min following i.[illegible]jection the occurrence [illegible]onic seizures was ≥66% of vehicle [score: ≥0.0559 compared to vehicle; Fig. 1(c)]. Co-injection of 3 nmol (R)-3,4-DCPG and 10 nmol (S)-3,4-DCPG produced an inhibition of sound-induced clonic seizures greater than either compound [illegible]ne at 60 min [Fig. 1(c)], where the score [illegible]so sig[illegible]ficantly different from that of the vehicle ([illegible]=0.0047). (RS)-3,4-DCPG also produced an inhibition of sound-induced clonic sei[illegible] when administered systemically whereby the ED_{50}=86 [74–101] mg/kg, i.p. with a steep dose response curve [Fig. 1(b)].

The 3,4-DCPG compounds produced a dose-dependent motor impairment when tested on the rotarod — ED_{50} (nmol, i.c.v.): 2.79 [1.46-[illegible] ((S)-3,4-DCPC [illegible]≤4.7 [0.8–25.2] ((RS)-3,4-DCPC [illegible]≤6.06 [3.84–9.57] ((R)-3,4-DCPG). The therapeutic indices of the 3,4-DCP[illegible] [illegible]ompounds were calculated (rotarod locomotor d ficit ED_{50}/anticonvul[illegible] ED_{50}): 1175, (RS)-3,4-DCP[illegible] >>25, (S)-3,4-DCP[illegible] >16, (R)-3,4-DCPG.

Following the observation that the racemate provided greater than additive inhibition of sound-induced seizures compared with the isomers given alone, the $mGlu_8$ agonist (RS)-PPG (0.03–3 nmol; (Chapman et al., 1999)) was co-injected i.c.v. with (R)-3,4-DCPG (0.001–0.1 nmol). Treatment with these compounds produced an [illegible]bition of sound-induced seizures with a similar profile to that of (RS)-3,4-DCPG, whereby the ED_{50} values for the co-injection were 0.011 [0.0017–0.076] nmol for (R)-3,4-DCPG or 0.34 [0.052–2.27] nmol for (RS)-PPG (from Chapman et al., 1999) [Fig. 1(d)]. Similarly, co-injection of (R)-3,4-DCPG (0.01–0[illegible]nol) with (RS)-PPG (0.3–3 nmol) produced a sig[illegible]ficant inhibition of seizure score compared to the [illegible]istration of similar doses of (R)-3,4-DCPG alone ([illegible]≤0.0068).

Sound-induced seizures in DBA/2 mice are an established animal model of generalised epilepsy in humans, such that all effective anti-epileptic drugs in humans potently inhibit seizures in DBA/2 mice. The 3,4-DCPG compounds inhibited sound-induced clonic seizures with the following order [illegible]otency (i.c.v.) (RS)-3,4-DCP >>(S)-3,4-DCP >(R)-3,4-DCPG. Based on their respective ED_{50} values (RS)-3,4-DCPG is approximately 30- and 100-fold more potent at inhibiting seizures than either (S)-3,4-DCPG and (R)-3,4-DCPG, respectively; (S)-3,4-DCPG is approximately 3-fold more potent than (R)-3,4-DCPG in this regard. The concentration response curve for the depression of the fast component of the dorsal root-evoked ventral root potential by (S)-3,4-DCPG in the neonatal rat spin[illegible]d was [illegible]sic (Thomas [illegible]., 2001). The unidentified weak [illegible]finity component ~300-fold difference in EC_{50} values) of this concentration response curve to (S)-3,4-DCPG cannot be ignored, however no biphasic response was observed with this compound in the present study. While the weak antagonism of NMDA and kainate receptors of (R)- and (RS)-3,4-DCPG (Thomas et al., 1997) is unlikely to make a sig[illegible]ficant difference to the relative potencies demonstrated here. Interestingly, despite the greater potency of (RS)-3,4-DCPG, the racemate did not impair motor performance beyond that experienced with either of the isomers.

At doses with which (R)-3,4-DCPG did not inhibit sound-induced clonic seizures, the racemate provided greater inhibition of seizures than (S)-3,4-DCPG alone at a similar dose. Since (R)-3,4-DCPG possesses negligible activity at $mGlu_8$, these findings suggest that a potentiation of the inhibition of sound-induced seizures is occurring, and results from the activation of [illegible]Glu_8 and antagonism at AMPA receptors. To co firm this

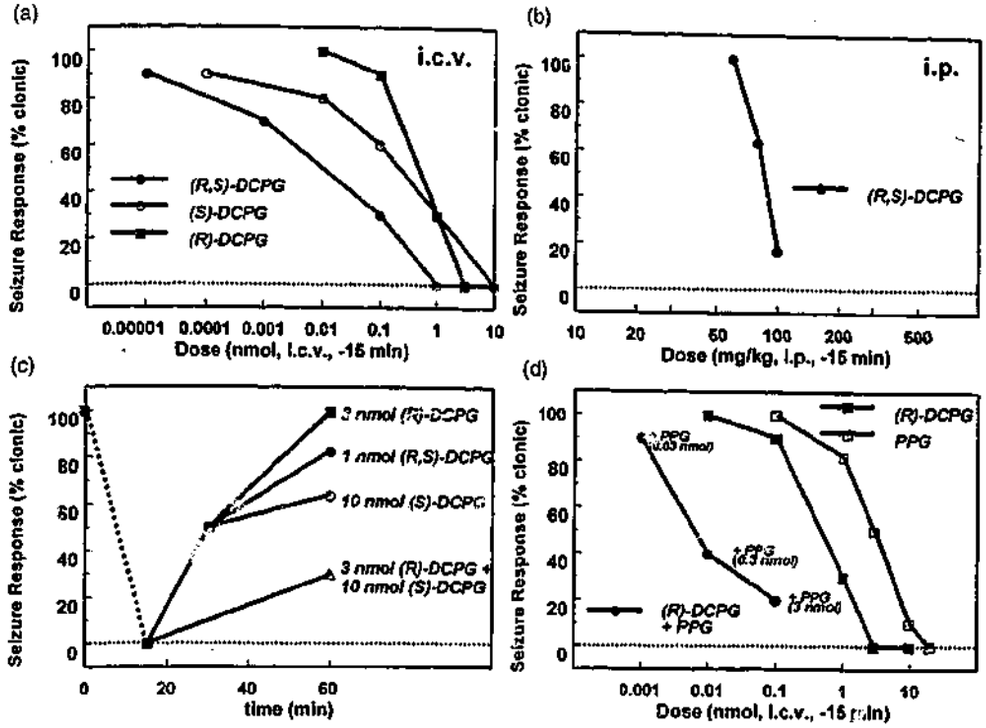


Fig. 1. Sound-induced clonic seizures in DBA/2 mice. (a) Dose-dependent inhibition of seizures following i.c.v. injection of 3,4-DCPG. =10 per data point. (b) Dose-dependent inhibition of seizures by i.p. injection of (RS)-3,4-DCPG. =6 per data point. (c) Time-dependent loss of anticonvulsant activity of 3,4-DCPG (i.c.v.). =10 per data point. (d) Potentiation of the inhibition of sound-induced clonic seizures by co-administration of (R)-3,4-DCPG and (RS)-PPG compared to either compound alone. =10 per data point. (RS)-PPG alone data from Chapman et al. (1999).

potentiating mechanism, the $mGlu_8$ agonist (RS)-PPG was co-injected with the AMPA receptor antagonist (R)-3,4-DCPG. Again, at doses with which (R)-3,4-DCPG did not provide inhibition of sound-induced seizures, a greater than additive inhibition of seizures was obtained by co-injection of the compounds than with (RS)-PPG alone. Co-injection of the compounds produced an approximate 10- and 35-fold greater inhibition of sound-induced seizures than either (RS)-PPG and (R)-3,4-DCPG, respectively.

This study has shown that a potentiation of the inhibition of sound-induced seizures is obtained by activation of $mGlu_8$ and antagonism at AMPA receptors, and that this presents a novel and effective strategy for the amelioration of seizures. Furthermore, since (RS)-3,4-DCPG is systemically active, the racemate is a novel and effective compound for the investigation of the interaction of $mGlu_8$ activation and AMPA receptor antagonism in models of epilepsy.

Acknowledgements

We thank the Epilepsy Research Foundation (UK) and the Fund for Epilepsy (UK) for providing financial support for this study. R. Moldrich is partly funded by the Centre for Neurosciences (Melbourne, Australia) and P. Beart by NH and MRC (Australia).

References

Bleakman, D., Lodge, D., 1998. Neuropharmacology of AMPA and kainate receptors. Neuropharmacology 37, 1187–1204.

Cartmell, J., Schoepp, D.D., 2000. Regulation of neurotransmitter release by metabotropic glutamate receptors. Journal of Neurochemistry 75, 889–907.

Chapman, A.G., Smith, S.E., Meldrum, B.S., 1991. The anticonvulsant effect of the non-NMDA antagonists, NBQX and GYKI 52466. Epilepsy Research 9, 92–96.

Chapman, A.G., Nanan, K., Yip, P., Meldrum, B.S., 1999. Anticonvulsant activity of a metabotropic glutamate receptor 8 preferential agonist, (RS)-4-phosphonophenylglycine. European Journal of Pharmacology 383, 23–27.

Chapman, A.G., Nanan, K., Williams, M., Meldrum, B.S., 2000. Anticonvulsant activity of two metabotropic glutamate Group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacology 39, 1567–1574.

Ghauri, M., Chapman, A., Meldrum, B.S., 1996. Convulsant and anticonvulsant actions of agonists and antagonists of group III mGluRs. NeuroReport 7, 1469–1474.

Meldrum, B.S., Craggs, M.D., Durmuller, N., Smith, S.E., Chapman,

A.G., 1992. The effects of AMPA receptor antagonists on kindled seizures and on [illegible] epilepsy in rodents and primates. Epilepsy Research 9 (Suppl), 307–311.

Thomas, N.K., Clayton, P., Jane, D.E., 1997. Dicarboxyphenylglycines antagonize AMPA—but not kainate-induced depolarizations in neonatal rat motoneurones. European Journal of Pharmacology 338, 111–116.

Thomas, N.K., Wright, R.A., Howson, P.A., Kingston, A.E., Schoepp, D.D., Jane, D.E., 2001. (S)-3,4-DCPG a potent and selective mGlu8a receptor agonist activates metabotropic glutamate receptors on primary afferent terminals in the neonatal rat spinal cord. Neuropharmacology 40, 311–318.

Appendix VI: Cortical and Striatal Neuronal Culture

Pregnant Swiss White mice (Monash University Central Animal House) of gestation day 14-16 were sacrificed by cervical dislocation. Under sterile conditions, the foetuses were removed and decapitated, their brains were dissected free and the cerebral cortices or neostriatum were microdissected under a dissection microscope (Industrial and Scientific Supply Co.). The dissection was conducted over ice in Hank's balance salt solution (HBSS; 137 mM NaCl, 5.37 mM KCl, 4.10 mM $NaHCO_3$ 0.44 mM KH_2PO_4 0.13 mM $NaHPO_4$, 10 mM HEPES, 1 mM pyruvate, 13 mM D(+)glucose and 0.001 g/L phenol red) containing 3 mg/ml bovine serum albumin (BSA) and 1.2 mM $MgSO_4$ (pH 7.4). Care was taken to remove the meninges and blood vessels. The tissue was broken into small pieces using a plastic pipette tip and briefly centrifuged (Labofuge 400e, Heraeus Instruments) at 1000 g x force to collect the fragments. The pellet of fragments was resuspended in warm (37°C) HBSS (with 3 mg/ml BSA and 1.2 mM $MgSO_4$) containing trypsin (0.2 mg/ml) and deoxyribonuclease I (DNase I, 880 U/ml) and incubated for 5 min at 37°C in a shaking water bath (Gallenkamp). The digestion of the fragments was terminated by addition of an equal volume of HBSS (with 3 mg/ml BSA) containing trypsin inhibitor (83.2 µg/ml, DNase I 880 U/ml) and $MgSO_4$ (1.22 mM) and centrifugation for 5 min at 1000 g x force. The supernatant was aspirated and HBSS containing trypsin inhibitor (0.52 mg/ml), DNase I (880 U/ml) and $MgSO_4$ (2.7 mM) was added to the pellet. The tissue was dissociated by trituration (15 strokes with a 24 gauge needle) and spun down for 5 min at 1000 g x force. The supernatant was aspirated and the cells were resuspended in Neurobasal™ medium (NBM; see Appendix I) containing 2% B27 supplement (see Appendix II), 100 U/ml penicillin and 100 µg/ml streptomycin, 0.5 mM L-glutamine and 10% dialysed foetal calf serum (dFCS), henceforth referred to as full NBM. The cell density of the suspension and culture yield were established by repeated cell counting on heamocytrometer chambers. Cells were seeded in Nunc™ (Denmark) 24- or 96-well plates at densities of 0.3×10^6 or 0.12×10^6 cells/well respectively, designated zero days *in vitro* (0 div). Plates were previously coated with poly-D-lysine (50 µg/ml), which was removed after incubation overnight at 37°C, to aid cell adherence. After 24 h (1 div), the full NBM was replaced with dFCS-free full NBM (2.5% B27 supplement). Half of the serum-free full NBM was replaced each 3-4 div. Cells were maintained in a humidified CO_2 incubator (5% $CO_{2,\ 8/5\%}$

O_2, 37°C; Forma Scientific) and examined using inverted, phase-contrast microscopy (Olympus, IMT-2). Photographs (Kodacolor Gold 100 ISO film) were taken to document the morphology of cultures undergoing insult. All experiments were carried out at room temperature (~ 21°C unless otherwise indicated).

Appendix VII: Cerebellar Granule Cell Culture

Swiss White mice pups (Monash University Central Animal House) of postnatal day 7 were sacrificed by cervical dislocation. Under sterile conditions, brains were dissected free and the cerebellar cortex was microdissected under a dissection microscope (Industrial and Scientific Supply Co.). The dissection was conducted over ice in Hank's balance salt solution (HBSS; 137 mM NaCl, 5.37 mM KCl, 4.10 mM $NaHCO_3$ 0.44 mM KH_2PO_4 0.13 mM $NaHPO_4$, 10 mM HEPES, 1 mM pyruvate, 13 mM D(+)glucose and 0.001 g/L phenol red) containing 3 mg/ml bovine serum albumin (BSA) and 1.2 mM $MgSO_4$ (pH 7.4). Care was taken to remove the meninges and blood vessels. The tissue was broken into small pieces using a plastic pipette tip and briefly centrifuged (Labofuge 400e, Heraeus Instruments) at 1000 g x force to collect the fragments. The pellet of fragments was resuspended in warm (37°C) HBSS (with 3 mg/ml BSA and 1.2 mM $MgSO_4$) containing trypsin (0.2 mg/ml) and deoxyribonuclease I (DNase I, 1600 U/ml) and incubated for 30 min at 37°C in a shaking water bath (Gallenkamp). The digestion of the fragments was terminated by addition of an equal volume of HBSS (with 3 mg/ml BSA) containing trypsin inhibitor (83.2 µg/ml, DNase I 1600 U/ml) and $MgSO_4$ (1.22 mM) and centrifugation for 3 min at 1500 g x force. The supernatant was aspirated and HBSS containing trypsin inhibitor (0.52 mg/ml), DNase I (880 U/ml) and $MgSO_4$ (2.7 mM) was added to the pellet. The tissue was dissociated by trituration (15 strokes with a 24 gauge needle) and spun down for 3 min at 1500 g x force. The supernatant was aspirated and the cells were resuspended in Neurobasal™ medium (NBM; see Appendix I) containing 2% B27 supplement (see Appendix II), 100 U/ml penicillin and 100 µg/ml streptomycin, 0.5 mM L-glutamine and 10% dialysed foetal calf serum (dFCS) (full NBM). The K^+ concentration was raised to 25 mM to prevent cell death. The cell density of the suspension and culture yield were established by

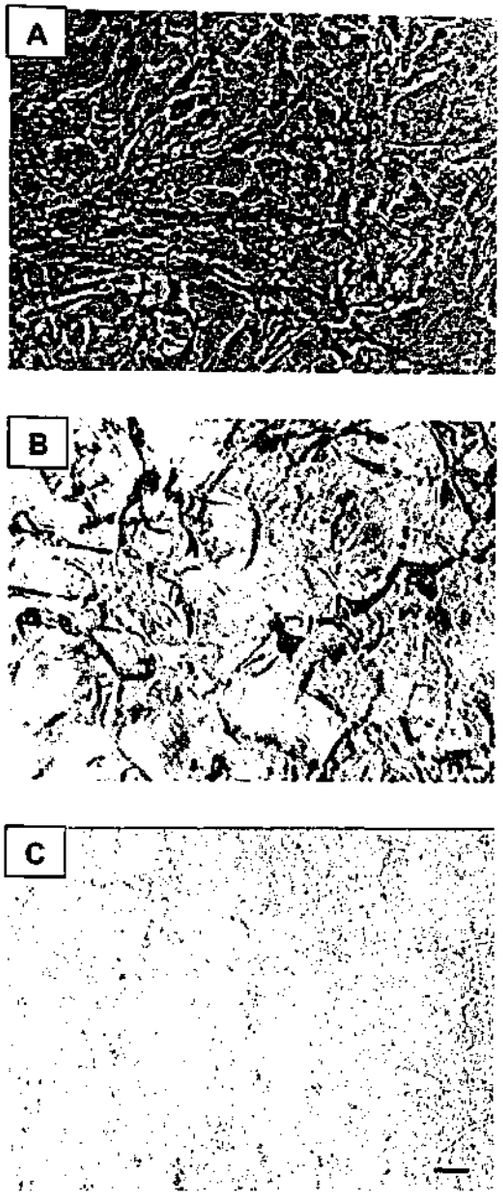
repeated cell counting on heamocytrometer chambers. Cells were seeded in Nunc™ (Denmark) 24- or 96-well plates at densities of 0.2 x 10^6 or 0.1 x 10^6 cells/well respectively, designated zero days *in vitro* (0 div). Plates were previously coated with poly-D-lysine (50 µg/ml), which was removed after incubation overnight at 37°C, to aid cell adherence. After 24 h (1 div), the full NBM was replaced with dFCS-free full NBM (25 mM K^+ and 2.5% B27 supplement). At this stage also 2 µg/ml of aphidicolin was included to prevent proliferation of astrocytes. Half of the serum-free full NBM (with 25 mM K^+ and aphidicolin) was replaced each 3-4 div. Cells were maintained in a humidified CO_2 incubator (5% CO_2, 8.5% O_2; 37°C;Forma Scientific) and examined using inverted, phase-contrast microscopy (Olympus, IMT-2). Photographs (Kodacolor Gold 100 ISO film) were taken to document the morphology of cultures undergoing insult. All experiments were carried out at room temperature (~ 21°C unless otherwise indicated).

Appendix VIII: Astrocyte Culture

Swiss White mice pups (Monash University Central Animal House) of postnatal day 1-2 were sacrificed by cervical dislocation. Under sterile conditions, their brains were dissected free and the forebrain was microdissected under a dissection microscope (Industrial and Scientific Supply Co.). The dissection was conducted over ice in Hank's balance salt solution (HBSS; 137 mM NaCl, 5.37 mM KCl, 4.10 mM $NaHCO_3$ 0.44 mM KH_2PO_4 0.13 mM $NaHPO_4$, 10 mM HEPES, 1 mM pyruvate, 13 mM D(+)glucose and 0.001 g/L phenol red) containing 3 mg/ml bovine serum albumin (BSA) and 1.2 mM $MgSO_4$ (pH 7.4). Care was taken to remove the olfactory bulb, meninges and blood vessels. The tissue was broken into small pieces using a plastic pipette tip and briefly centrifuged (Labofuge 400e, Heraeus Instruments) at 1000 g x force to collect the fragments. The pellet of fragments was resuspended in warm (37°C) HBSS (with 3 mg/ml BSA and 1.2 mM $MgSO_4$) containing trypsin (0.2 mg/ml) and deoxyribonuclease I (DNase I, 880 U/ml) and incubated for 5 min at 37°C in a shaking water bath (Gallenkamp). The digestion of the fragments was terminated by addition of an equal volume of HBSS (with 3 mg/ml BSA) containing trypsin inhibitor (83.2 µg/ml, DNase I 880

U/ml) and $MgSO_4$ (1.22 mM) and centrifugation for 5 min at 1000 g x force. The supernatant was aspirated and HBSS containing trypsin inhibitor (0.52 mg/ml), DNase I (880 U/ml) and $MgSO_4$ (2.7 mM) was added to the pellet. The tissue was dissociated by trituration (15 strokes with a 24 gauge needle) and spun down for 5 min at 1000 g x force. The supernatant was aspirated and the cells were resuspended in Astrocyte Medium (AM) consisting of Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, penicillin/streptomycin (100 U/ml/100 μg/ml) and Fungizone® (amphotericin B, 1 μg/ml). In general 10 ml of this solution, containing the forebrain tissue of two pups, was deposited in 75 ml Nunc flasks (with filter lids) and maintained at 36°C and 5% CO_2. Astrocytes were grown to confluency with twice weekly medium changes of AM (approx. 12-14 div). Astrocytes were removed from the flasks and separated from microglia by overnight shaking at 150 rpm (Ratek Orbital Mixer; 37°C), followed by a further shake in warmed AM (150 rpm, 2 h, 37°C). Cells were then allowed to incubate at room temperature with 10 mM EDTA in the flasks before the cell suspension was transferred to centrifuge tubes and spun down at 1500 g x force (5 min). Astrocytes were replated in AM in Nunc multiwell plates at 3.0×10^4 or 8.0×10^4 cells/well for calcium assay (96-well plates) or cAMP assay (24-well plates) experiments. Astrocytes were grown to confluence with twice weekly medium changes of AM at 36°C and 5% CO_2, such that at the time of the experiments astrocytes were 24–28 div.

APPENDIX IX: Photos of astrocyte cultures



A. Representative phase-contrast photomicrograph of cultured astrocytes at 24 div. Astrocytes typically display a flat, polygonal morphology.

B. Representative brightfield photomicrograph of cultured astrocytes at 24 div following immunocytochemistry using anti-GFAP (glial marker).

C. Representative brightfield photomicrograph of cultured astrocytes at 24 div following immunocytochemistry using anti-MAP-2 (neuronal marker). Astrocyte cultures contain no positive labelling of neurones. Scale bar = 30 µm.

Appendix X: Measurements of Intracellular Calcium Concentration

(Protocol for cultures in a 96-well plate). Cells were washed with 100 μl of warmed HEPES-buffered saline (HBS: 120 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 6 mM glucose; pH 7.4). Cells were then incubated with 50 μl of warmed Fluo-3/AM solution (10 μM Fluo-3/AM in HBS containing 1% v/v DMSO and 0.2% w/v Pluronic F-127), at room temperature for 15 min in the dark (and thereafter if possible). A further 50 μl of warmed HBS was added and cells were incubated at 37°C for 1 h. This dye-loading solution was aspirated and cells washed with 100 μl of warmed HBS containing 1 mM furosemide (HBSF). New HBSF (50 μl) containing drug treatments or control was added to the cells and the fluorescence measured immediately using the Ascent Fluoroskan for approx. 5 min (excitation/emission: 485/535). The maximum fluorescence (Fmax) was determined after this reading by aspirating the treatment solution and adding 50 μl of HBSF containing 10 μM of A23187 for approx. 30 min at 37°C then read in the Ascent Fluoroskan. The minimum fluorescence (Fmin) was taken after Fmax, whereby an additional 50 μl of 2mM $CuCl_2$ (in 0.9% w/v NaCl) with 10 μM A23187 was added and read in the Ascent Fluoroskan 5 min later. Intracellular calcium concentration, $[Ca^{2+}]i = Kd\ (F-Fmin)/(Fmax-F)$ where Kd is 450nM. To diminish variable scatter of levels $[Ca^{2+}]i$ can be expressed as $\Delta = ([Ca]i-[Ca]ir) \times 100/[Ca]ir$ where $[Ca^{2+}]ir$ is under resting conditions (i.e. 5 mM KCl).

REFERENCES

References appear in alphabetical, then chronological order.

Abbott, A., Wigmore, M. A., Lacey, M. G., 1997. Excitation of rat subthalamic nucleus neurones in vitro by activation of a group I metabotropic glutamate receptor. Brain Research 766, 162-167.

Abdul-Ghani, A. S., Attwell, P. J., Singh Kent, N., Bradford, H. F., Croucher, M. J., Jane, D. E., 1997. Anti-epileptogenic and anticonvulsant activity of L-2-amino-4-phosphonobutyrate, a presynaptic glutamate receptor agonist. Brain Research 755, 202-212.

Abe, K., Saito, H., 1998. Characterization of *t*-butyl hydrogen peroxide toxicity in cultured rat cortical neurones and astrocytes. Journal of Pharmacology and Toxicology 83, 40-46.

Adamson, P., Hajimohammadreza, I., Brammer, M. J., Campbell, I. C., Meldrum, B. S., 1990. Presynaptic glutamate/quisqualate receptors: effects on synaptosomal free calcium concentrations. Journal of Neurochemistry 55, 1850-1854.

Akazawa, C., Ohishi, H., Nakajima, Y., Okamoto, N., Shigemoto, R., Nakanishi, S., Mizuno, N., 1994. Expression of mRNA of L-AP4-sensitive metabotropic glutamate receptors (mGluR4, mGluR6, mGluR7) in the rat retina. Neuroscience Letters 171, 52-54.

Allen, J. W., Ivanova, S. A., Fan, L., Espey, M. G., Basile, A. S., Faden, A. I., 1999. Group II metabotropic glutamate receptor activation attenuates traumatic neuronal injury and improves neurological recovery after traumatic brain injury. Journal of Pharmacology and Experimental Therapeutics 290, 112-120.

Allen, J. W., Vicini, S., Faden, A. I., 2001. Exacerbation of neuronal cell death by activation of group I metabotropic glutamate receptors: role of NMDA receptors and arachidonic acid release. Experimental Neurology 169, 449-460.

Anderson, K. J., Monaghan, D. T., Cangro, C. B., Namboodiri, M. A., Neale, J. H., Cotman, C. W., 1986. Localization of N-acetylaspartylglutamate-like immunoreactivity in selected areas of the rat brain. Neuroscience Letters 72, 14-20.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., Nicotera, P., 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15, 961-973.

Antoni, F. A., 2000. Molecular diversity of cyclic AMP signalling. Frontiers in Neuroendocrinology 21, 103-132.

Anwyl, R., 1999. Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Research Reviews 29, 83-120.

Araque, A., Li, N., Doyle, R. T., Haydon, P. G., 2000. SNARE protein-dependent glutamate release from astrocytes. Journal of Neuroscience 20, 666-673.

Aronica, E., Catania, M. V., Geurts, J., Yankaya, B., Troost, D., 2001. Immunohistochemical localization of group I and II metabotropic glutamate receptors in control and amyotrophic lateral sclerosis human spinal cord: upregulation in reactive astrocytes. Neuroscience 105, 509-520.

Aronica, E., Condorelli, D. F., Nicoletti, F., Dell'Albani, P., Amico, C., Balazs, R., 1993. Metabotropic glutamate receptors in cultured cerebellar granule cells: developmental profile. Journal of Neurochemistry 60, 559-565.

Aronica, E., van Vliet, E. A., Mayboroda, O. A., Troost, D., da Silva, F. H., Gorter, J. A., 2000. Upregulation of metabotropic glutamate receptor subtype mGluR3 and mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. European Journal of Neuroscience 12, 2333-2344.

Attwell, P. J. E., Kaura, S., Sigala, G., Bradford, H. F., Croucher, M. J., Jane, D. E., Watkins, J. C., 1995. Blockade of both epileptogenesis and glutamate release by (1S,3S)-ACPD, a presynaptic glutamate receptor agonist. Brain Research 698, 155-162.

Attwell, P. J. E., Kent, N. S., Jane, D. E., Croucher, M. J., Bradford, H. F., 1998a. Anticonvulsant and metabotropic glutamate release-inhibiting properties of the highly potent metabotropic glutamate receptor agonist (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV). Brain Research 805, 138-143.

Attwell, P. J. E., Koumentaki, A., Croucher, M. J. and Bradford, H. F., 1998b. Specific group II glutamate receptor activation inhibits the development of kindled epilepsy in rats. Brain Research 787, 286-291.

Auer, R., Siesjo, B. K., 1985. Status epilepticus in well oxygenated rats causes neuronal necrosis. Annals of Neurology 18, 281-290.

Awad, H., Hubert, G. W., Smith, Y., Levey, A. I., Conn, P. J., 2000. Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. Journal of Neuroscience 20, 7871-7879.

Baird, J. G., Challiss, R. A. J., Nahorski, S. R., 1991. Role for ionotropic and metabotropic glutamate receptors in quisqualate-stimulated inositol polyphosphate accumulation in rat cerebral cortex. Molecular Pharmacology 39, 745-753.

Baker, S. R., Goldsworthy, J., Harden, R. C., Salhoff, C. R., Schoepp, D. D., 1995. Enzymatic resolution and pharmacological activity of the enantiomers of 3,5-dihydroxyphenylglycine, a metabotropic glutamate receptor agonist. Bioorganic Medicinal Chemistry Letters 5, 223-228.

Balázs, R., Miller, S., Chun, Y., Cotman, C. W., 1998b. Receptor-coupled phospholipase C and adenylyl cyclase function with different calcium pools in astrocytes. Neuroreport 9, 1397-1401.

Balázs, R., Miller, S., Chun, Y., O'Toole, J., Cotman, C. W., 1998a. Metabotropic glutamate receptor agonists potentiate cyclic AMP formation induced by forskolin or β-adrenergic receptor activation in cerebral cortical astrocytes in culture. Journal of Neurochemistry 70, 2446-2458.

Balcar, V. J., Johnston, G. A., 1972. The structural specificity of the high affinity uptake of L-glutamate and L-aspartate by rat brain slices. Journal of Neurochemistry 19, 2657-2666.

Bao, W. L., Williams, A. J., Faden, A. I., Tortella, F. C., 2001. Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. Brain Research 922, 173-179.

Bargmann, C. I., Kaplan, J. M., 1998. Signal transduction in the Caenorhabditis elegans nervous system. Annual Review of Neuroscience 21, 279-308.

Batchelor, A. M., Knophel, T., Gasparini, F., Garthwaite, J., 1997. Pharmacological characterization of synaptic transmission through mGluRs in rat cerebellar slices. Neuropharmacology 36, 401-403.

Battaglia, G., Bruno, V., Ngomba, R. T., Di Grezia, R., Copani, A., Nicoletti, F., 1998. Selective activation of group-II metabotropic glutamate receptors is protective against excitotoxic neuronal death. European Journal of Pharmacology 356, 271-274.

Battaglia, G., Monn, J. A., Schoepp, D. D., 1997. In vivo inhibition of veratridine-evoked release of striatal excitatory amino acids by the group II metabotropic glutamate receptor agonist LY354740 in rats. Neuroscience Letters 229, 161-164.

Beart, P. M., 1976. The autoradiographic localization of L-[^{3}H]glutamate in synaptosome preparations. Brain Research 103, 350-355.

Beart, P. M., Mercer, L. D., Jones, N. M., 1999. Immunohistochemistry with an antibody directed at mGluR3. Neuropharmacology 38, A5.

Beart, P. M., Summers, R. J., Stephenson, J. A., Christie, M. J., 1994. Excitatory amino acid projections to the nucleus of the solitary tract in the rat: a retrograde transport study utilizing D-[3H]aspartate and [3H]GABA. Journal of the Autonomic Nervous System 50, 109-122.

Beckstrom, H., Julsrud, L., Haugeto, O., Dewar, D., Graham, D. I., Lehre, K. P., Storm-Mathisen, J., Danbolt, N. C., 1999. Interindividual differences in the levels of the glutamate transporters GLAST and GLT, but no clear correlation with Alzheimer's disease. Journal of Neuroscience Research 55, 218-229.

Behrens, M. M., Strasser, U., Heidinger, V., Lobner, D., Yu, S.-P., McDonald, J. W., Won, M., Choi, D. W., 1999. Selective activation of group II mGluRs with LY354740 does not prevent neuronal excitotoxicity. Neuropharmacology 38, 1621-1630.

Bellomo, M., Giuffrida, R., Palermi, A., Sapienza, S., 1998. Excitatory amino acids as neurotransmitters of corticalstriatal projections: immunocytochemical evidence in the rat. Archives of Italian Biology 136, 215-223.

Ben-Ari, Y., 1981. Epilepsy: changes in local glucose consumption and brain pathology produced by kainic acid. In, Glutamate as a neurotransmitter - advances in biochemical pyschopharmacology. Eds: DiChiara, G., Gessa, G. L. Raven Press, New York. pp. 403.

Ben-Ari, Y., 1985. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14, 375-403.

Bengzon, J., Mohapel, P., Ekdahl, C. T., Lindvall, O., 2002. Neuronal apoptosis after brief and prolonged seizures. Progress in Brain Research 135, 111-119.

Berkovic, S., 1985. Epilepsies with absence: classification and genetics. In, Grey areas in epilepsy. Eds: Vajda, F. J. E., Donnan, G. A. York Press, Melbourne. pp. 17-24.

Berthele, A., Platzer, S., Laurie, D. J., Weis, S., Sommer, B., Zieglgansberger, W., Conrad, B., Tolle, T. R., 1999. Expression of metabotropic glutamate receptor subtype mRNA (mGluR1-8) in human cerebellum. NeuroReport 10, 3861-3867.

Besong, G., Battaglia, G., D'Onofrio, M., Di Marco, R., Ngomba, R. T., Storto, M., Castiglione, M., Mangano, K., Busceti, C. L., Nicoletti, F. R., Bacon, K., Tusche, M., Valenti, O., Conn, P. J., Bruno, V., Nicoletti, F., 2002. Activation of group III metabotropic glutamate receptors inhibits the production of RANTES in glial cell cultures. Journal of Neuroscience 22, 5403-5411.

Bettler, B., Mulle, C., 1995. Review: neurotransmitter receptors. II. AMPA and kainate receptors. Neuropharmacology 34, 123-139.

Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B. L., Pozzan, T., Volterra, A., 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391, 281-285.

Birrell, G. J., Gordon, M. P., Marcoux, F. W., 1993. (1S,3R)-1-Aminocyclopentane-1,3-dicarboxyli acid attenuates *N*-methyl-D-aspartate-induced neuronal cell death in cortical cultures via a reduction in delayed Ca^{2+} accumulation. Neuropharmacology 32, 1351-1358.

Bladin, P. F., 1985. Absences - pure culture or mixed bag? In, Grey areas in epilepsy. Eds: Vajda, F. J. E., Donnan, G. A. York Press, Melbourne. pp. 11-16.

Blahos, J., 2nd, Mary, S., Perroy, J., de Colle, C., Brabet, I., Bockaert, J., Pin, J.-P., 1998. Extreme C terminus of G protein alpha-subunits contains a site that discriminates between Gi-coupled metabotropic glutamate receptors. Journal of Biological Chemistry 273, 25765-25769.

Blanc, E., Vignes, M., Recasens, M., 1995. Protein kinase C differently regulates quisqualate- and 1S,3R-trans aminocyclopentane dicarboxylate-induced phosphoinositide hydrolysis during in vitro development of hippocampal neurons. Neurochemistry International 26, 623-633.

Blanchard, B. J., Thomas, V. L., Ingram, V. M., 2002. Mechanism of membrane depolarization caused by the Alzheimer Abeta1-42 peptide. Biochemical and Biophysical Research Communications 293, 1197-1203.

Blumcke, I., Becker, A. J., Klein, C., Scheiwe, C., Lie, A. A., Beck, H., Waha, A., Friedl, M. G., Kuhn, R., Emson, P., Elger, C., Wiestler, O. D., 2000. Temporal lobe epilepsy associated upregulation of metabotropic glutamate receptors: Correlated changes in mGluR1 mRNA and protein expression in experimental animals and human patients. Journal of Neuropathology and Experimental Neurology 59, 1-10.

Blumcke, I., Behle, K., Malitschek, B., Kuhn, R., Knopfel, T., Wolf, H. K., Wiestler, O. D., 1996. Immunohistochemical distribution of metabotropic glutamate receptor subtypes mGluR1b, mGluR2/3, mGluR4a and mGluR5 in human hippocampus. Brain Research 736, 217-226.

Bockaert, J., Pin, J.-P., 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO Journal 18, 1723-1729.

Bockaert, J., Pin, J.-P., Fagni, L., 1993. Metabotropic glutamate receptors: an original family of G protein-coupled receptors. Fundamental and Clinical Pharmacology 7, 473-485.

Bond, A., Jones, N. M., Hicks, C. A., Whiffin, G. M., Ward, M. A., O'Neill, M. F., Kingston, A. E., Monn, J. A., Ornstein, P. L., Schoepp, D. D., Lodge, D., 2000. O'Neill, M.J. Neuroprotective effects of LY379268, a selective mGlu2/3 receptor agonist: investigations into possible mechanism of action in vivo. Journal of Pharmacology and Experimental Therapeutics 294, 800-809.

Bond, A., O'Neill, M. J., Hicks, C. A., Monn, J. A., Lodge, D., 1998. Neuroprotective effects of a systemically active group II metabotropic glutamate receptor agonist LY354740 in a gerbil model of global ischaemia. NeuroReport 9, 1191-1193.

Bond, A., Ragumoorthy, N., Monn, J. A., Hicks, C. A., Ward, M. A., Lodge, D., O'Neill, M. J., 1999. LY379268, a potent and selective group II metabotropic glutamate receptor agonist, is neuroprotective in gerbil global, but not focal, cerebral ischemia. Neuroscience Letters 273, 191-194.

Bortolotto, Z. A., Bashir, Z. I., Davies, C. H., Collingridge, G. L., 1994. A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. Nature 368, 740-743.

Bortolotto, Z. A., Collingridge, G. L., 1998. Involvement of calcium/calmodulin-dependent protein kinases in the setting of a molecular switch involved in hippocampal LTP. Neuropharmacology 37, 535-544.

Bortolotto, Z. A., Collingridge, G. L., 2000. A role for protein kinase C in a form of metaplasticity that regulates the induction of long-term potentiation at CA1 synapses of the adult rat hippocampus. European Journal of Neuroscience 12, 4055-4062.

Bortolotto, Z. A., Fitzjohn, S. M., Collingridge, G. L., 1999. Roles of metabotropic glutamate receptors in LTP and LTD in the hippocampus. Current Opinion in Neurobiology 3, 299-304.

Bose, R., Pinsky, C., Glavin, G. B., 1990. Sensitive murine model and putative antidotes for behaviorial toxicosis from contaminated mussel extracts. Canada Diseases Weekly Report 16, 91-98.

Boudin, H., Craig, A. M., 2001. Molecular determinants for PICK1 synaptic aggregation and mGluR7a receptor coclustering: role of the PDZ, coiled-coil, and acidic domains. Journal of Biological Chemistry 276, 30270-30276.

Boudin, H., Doan, A., Xia, J., Shigemoto, R., Huganir, R. L., Worley, P., Craig, A. M., 2000. Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron 28, 485-497.

Bougeois, B. F. D., 1998. New antiepileptic drugs. Archives of Neurology 55, 1181-1183.

Brabet, I., Mary, S., Bockaert, J., Pin, J.-P., 1995. Phenylglycine derivatives discriminate between mGluR1- and mGluR5-mediated responses. Neuropharmacology 34, 895-903.

Brabet, I., Parmentier, M.-L., De Colle, C., Bockaert, J., Acher, F., Pin, J.-P., 1998. Comparative effect of L-CCGI, DCG-IV and gama-carboxy-L-glutamate on all cloned metabotropic glutamate receptor subtypes. Neuropharmacology 37, 1043-1051.

Bradley, S. R., Marino, M. J., Wittmann, M., Rouse, S. T., Awad, H., Levey, A. I., Conn, P. J., 2000. Activation of group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia Nigra pars reticulata. Journal of Neuroscience 20, 3085-3094.

Brakeman, P. R., Lanahan, A. A., O'Brien, R., Roche, K., Barnes, C. A., Huganir, R. L., Worley, P. F., 1997. Homer: a protein that selectively binds metabotropic glutamate receptors. Nature 386, 284-288.

Breakwell, N. A., Rowan, M. J., Anwyl, R., 1996. Metabotropic glutamate receptor dependent EPSP and EPSP-spike potentiation in area CA1 of the submerged rat hippocampal slice. Journal of Neurophysiology 76, 3126-3135.

Brewer, G. J., 1995. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum and dentate gyrus. Journal of Neuroscience Research 42, 674-683.

Broman, J., Hassel, B., Rinvik, E., Ottersen, O. P., 2000. Biochemistry and anatomy of transmitter glutamate. In, Glutamate. Handbook of chemical neuroanatomy. Eds: Ottersen, O.P., Storm-Mathisen, J. Elsevier, Amsterdam. pp. 1-44.

Bronstein, J. M., Farber, D. B., Wasterlain, C. G., 1993. Regulation of type-II calmodulin kinase: functional implications. Brain Research Reviews 18, 135-147.

Bruno, V., Battaglia, G., Casabona, G., Copani, A., Caciagli, F., Nicoletti, F., 1998a. Neuroprotection by glial metabotropic glutamate receptors is mediated by transforming growth factor-beta. Journal of Neuroscience 18, 9594-9600.

Bruno, V., Battaglia, G., Copani, A., Giffard, R. G., Raciti, G., Raffaele, R., Shinozaki, H., Nicoletti, F., 1995a. Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. European Journal of Neuroscience 7, 1906-1913.

Bruno, V., Copani, A., Battaglia, G., Raffaele, R., Shinozaki, H., Nicoletti, F., 1994. Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death. European Journal of Pharmacology 256, 109-112.

Bruno, V., Copani, A., Bonanno, L., Knopfel, T., Kuhn, R., Roberts, P. J., Nicoletti, F., 1996. Activation of group III metabotropic glutamate receptors is neuroprotective in cortical cultures. European Journal of Pharmacology 310, 61-66.

Bruno, V., Copani, A., Knopfel, T., Kuhn, R., Casabona, G., Dell'Albani, P., Condorelli, D. F., Nicoletti, F., 1995b. Activation of metabotropic glutamate receptors coupled to inositol phospholipid hydrolysis amplifies NMDA-induced neuronal degeneration in cultured cortical cells. Neuropharmacology 34, 1081-1087.

Bruno, V., Sureda, F. X., Storto, M., Casabona, G., Caruso, A., Knopfel, T., Kuhn, R., Nicoletti, F., 1997. The neuroprotective activity of group-II metabotropic glutamate receptors requires new protein synthesis and involves a glial-neuronal signalling. Journal of Neuroscience 17, 1891-1897.

Bruno, V., Wroblewska, B., Wroblewski, J. T., Fiore, L., Nicoletti, F., 1998b. Neuroprotective activity of N-acetylaspartylglutamate in cultured cortical cells. Neuroscience 85, 751-757.

Bruton, R. K., Ge, J., Barnes, N. M., 1999. Group I mGlu receptor modulation of dopamine release in the rat striatum in vivo. European Journal of Pharmacology 369, 175-181.

Buisson, A., Choi, D. W., 1995. The inhibitory mGluR agonist, *S*-4-carboxyl-3-hydroxy-phenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. Neuropharmacology 34, 1081-1087.

Buisson, A., Yu, S. P., Choi, D. W., 1996. DCG-IV selectively attenuates rapidly triggered NMDA-induced neurotoxicity in cortical neurons. European Journal of Neuroscience 8, 138-143.

Burger, P. M., Mehl, E., Cameron, P. L., Maycox, P. R., Baumert, M., F., L., De Camilli, P., Jahn, R., 1989. Synaptic vesicles immunoisolated from rat cerebral cortex contain high levels of glutamate. Neuron 3, 715-720.

Burgess, D. L., Biddlecome, G. H., McDonough, S. I., Diaz, M. E., Zilinski, C. A., Bean, B. P., Campbell, K. P., Noebels, J. L., 1999. Beta subunit reshuffling modifies N- and P/Q-type Ca^{2+} channel subunit compositions in lethargic mouse brain. Molecular and Cellular Neuroscience 13, 293-311.

Burke, J. P., Hablitz, J. J., 1994. Metabotropic glutamate receptor activation decreases epileptiform activity in rat neocortex. Neuroscience Letters 174, 29-33.

Cai, Z., Saugstad, J. A., Sorensen, S. D., Ciombor, K. J., Zhang, C., Schaffhauser, H., Hubalek, F., Pohl, J., Duvoisin, R. M., Conn, P. J., 2001. Cyclic AMP-dependent protein kinase phosphorylates group III metabotropic glutamate receptors and inhibits their function as presynaptic receptors. Journal of Neurochemistry 78, 756-766.

Calabresi, P., Centonze, D., Pisani, A., Bernadi, G., 1999. Metabotropic glutamate receptors and cell-type-specific vulnerability in the striatum: implication for ischemia and Huntington's Disease. Experimental Neurology 158, 97-108.

Camon, L., Vives, P., de Vera, N., Martinez, E., 1998. Seizures and neuronal damage induced in the rat by activation of group I metabotropic glutamate receptors with their selective agonist 3,5-dihydroxyphenylglycine. Journal of Neuroscience Research 51, 339-348.

Campbell, V., Berrow, N. S., Fitzgerald, E. M., Brickley, K., Dolphin, A. C., 1995. Inhibition of the interaction of G protein G(o) with calcium channels by the calcium channel beta-subunit in rat neurones. Journal of Physiology (London) 485, 365-372.

Cao, C. Q., Tse, H.-W., Jane, D. E., Evans, R. H., Headley, P. M., 1997a. Antagonism of mGlu receptors and potentiation of EPSCs at rat spinal motoneurones in vitro. Neuropharmacology 36, 313-318.

Cao, C. Q., Tse, H.-W., Jane, D. E., Evans, R. H., Headley, P. M., 1997b. Metabotropic glutamate receptor antagonists, like $GABA_B$ antagonists potentiate dorsal root-evoked excitatory synaptic transmission at neonatal rat spinal motoneurons in vitro. Neuroscience 78, 243-250.

Carroll, F. Y., Stolle, A., Beart, P. M., Voerste, A., Brabet, I., Mauler, F., Joly, C., Antoniceck, H., Bockaert, J., Muller, T., Pin, J.-P., Prezeau, L., 2001. BAY36-7620: A potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. Molecular Pharmacology 59, 965-973.

Cartmell, J., Curtis, A. R., Kemp, J. A., Kendall, D. A., Alexander, S. P. H., 1993a. Subtypes of metabotropic excitatory amino acid receptor distinguished by stereoisomers of the rigid glutamate analogue, 1-aminocyclopentanedicarboxylate. Neuroscience Letters 153, 107-110.

Cartmell, J., Goepfert, F., Knoflach, F., Pink, J. R., Bleuel, Z., Richards, J. G., Schaffhauser, H., Kemp, J. A., Wichmann, J., Mutel, V., 1998. Effect of metabotropic glutamate receptor activation on receptor-mediated cyclic AMP responses in primary cultures of rat striatal neurones. Brain Research 791, 191-199.

Cartmell, J., Kemp, J. A., Alexander, S. P. H., Hill, S. J., Kendall, D. A., 1992. Inhibition of forskolin-stimulated cyclic AMP formation by 1-aminocyclopentane-trans-1,3-dicarboxylate in guinea-pig cerebral cortical slices. Journal of Neurochemistry 58, 1964-1966.

Cartmell, J., Kemp. J. A., Alexander, S. P. H., Kendall, D. A., 1993b. Endogenous adenosine regulates the apparent efficacy of 1-aminocyclopentyl-1*S*,3*R*-dicarboxylate inhibition of forskolin-stimulated cyclic AMP accumulation in rat cerebral cortical slices. Journal of Neurochemistry 60, 780-782.

Cartmell, J., Kemp, J. A., Alexander, S. P. H., Shinozaki, H., Kendall, D. A., 1994. Modulation of cyclic AMP formation by putative metabotropic receptor agonists. British Journal of Pharmacology 111, 364-369.

Cartmell, J., Schaffhauser, H., Wichmann, J., Mutel, V., 1997. mGluR-evoked augmentation of receptor-mediated cyclic AMP formation in neonatal and adult rat striatum. British Journal of Pharmacology 121, 1263-1268.

Cartmell, J., Schoepp, D. D., 2000. Regulation of neurotransmitter release by metabotropic glutamate receptors. Journal of Neurochemistry 75, 889-907.

Casabona, G., Genazzani, A. A., Di Stephano, M., Sortino, M. A., Nicoletti, F., 1992. Developmental changes in the modulation of cyclic AMP formation by the metabotropic glutamate receptor agonist 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid in brain slices. Journal of Neurochemistry 59, 1161-1163.

Cepeda, C., Colwell, C. S., Itri, J. N., Chandler, S. H., Levine, M. S., 1998. Dopaminergic modulation of NMDA-induced whole cell currents in neostriatal neurons in slices: contribution of calcium conductances. Journal of Neurophysiology 79, 82-94.

Cha, J. H., Kosinski, C. M., Kerner, J. A., Alsdorf, S. A., Mangiarini, L., Davies, S. W., Penney, J. B., Bates, G. P., Young, A. B., 1998. Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human huntington disease gene. Proceedings of the National Academy of Sciences of the United States of America 95, 6480-6485.

Chapman, A. G., Elwes, R. D. C., Millan, M. H., Polkey, C. E., Meldrum, B. S., 1996. Role of glutamate and aspartate in epileptogenesis; contribution of microdialysis studies in animals and man. Progressive Nature of Epileptogenesis (Epilepsy Research Supplement 12) 12, 239-246.

Chapman, A.G., Meldrum, B.S., 1987. Epilepsy prone mice: genetically determined sound-induced seizures. In, Neurotransmitters and epilepsy. Eds: Jobe, P. C., Laird II, H. E. Humana Press, Clifton. pp. 9–40.

Chapman, A. G., Nanan, K., Williams, M., Meldrum, B. S., 2000. Anticonvulsant activity of two metabotropic glutamate Group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethylnl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacology 39, 1567-1574.

Chapman, A. G., Smith, S. E., Meldrum, B.S., 1991. The anticonvulsant effect of the non-NMDA antagonists, NBQX and GYKI 52466. Epilepsy Research 9, 92-96.

Chapman, A. G., Yip, P. K., Yap, J. S., Quinn, L. P., Tang, E., Harris, J. R., Meldrum, B. S., 1999. Anticonvulsant actions of LY367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((*RS*)-1-aminoindan-1,5-dicarboxylic acid). European Journal of Pharmacology 368, 17-24.

Charbardès, D., Imbert-Teboul, M., Elalouf, J-M., 1999. Functional properties of Ca^{2+}-inhibitable type 5 and type 6 adenylyl cyclases and role of Ca^{2+} increase in the inhibition of intracellular cAMP content. Cellular Signalling 11, 651-663.

Chavis, P., Fagni, L., Bockeart, J., Lansman, J. B., 1995. Modulation of calcium channels by metabotropic glutamate receptors in cerebellar granule cells. Neuropharmacology 34, 929-937.

Chavis, P., Fagni, L., Lansman, J. B., Bockaert, J., 1996. Functional coupling between ryanodine receptors and L-type Ca2+ channels in neurons. Nature 382, 719-722.

Chavis, P., Shinozaki, H., Bockaert, J., Fagni, L., 1994. The metabotropic glutamate receptor types 2/3 inhibit L-type calcium channels via a pertussis toxin-sensitive G-protein in cultured cerebellar granule cells. Journal of Neuroscience 14, 7067-7076.

Chen, W., Aoki, C., Mahadomrongkul, V., Gruber, C. E., Wang, G. J., Blitzblau, R., Irwin, N., Rosenberg, P. A., 2002. Expression of a variant form of the glutamate transporter GLT1 in neuronal cultures and in neurons and astrocytes in the rat brain. Journal of Neuroscience 22, 2142-2152.

Chenard, B. L., Menniti, F. S., 1999. Antagonists selective for NMDA receptors containing the NR2B subunit. Current Pharmaceutical Design 5, 381-404.

Cheung, N. S., Beart, P. M., Pascoe, C. J., John, C. A., Bernard, O., 2000. Human Bcl-2 protects against AMPA receptor-mediated apoptosis. Journal of Neurochemistry 74, 1613-1620.

Cheung, N. S., Carroll, F. Y., Lam, J. A., Beart, P. M., Giardina, S. F., 1998a. Kainate-induced apoptosis correlates with *c-Jun* activation in cultured cerebellar granule cells. Journal of Neuroscience Research 52, 69-82.

Cheung, N. S., Pascoe, C. J., Giardina, S. F., John, C. A., Beart, P. M., 1998b. Micromolar L-glutamate induces extensive apoptosis in an apoptotic-necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. Neuropharmacology 37, 1419-1429.

Cho, K., Brown, M. W., Bashir, Z. I., 2002. Mechanisms and physiological role of enhancement of mGlu5 receptor function by group II mGlu receptor activation in rat perirhinal cortex. Journal of Physiology 540, 895-906.

Cho, K., Kemp, N., Noel, J., Aggleton, J. P., Brown, M. W., Bashir, Z. I., 2000. A novel form of long-term depression in the perirhinal cortex. Nature Neuroscience 3, 150-156.

Choi, D. W., 1995. Calcium: still center-stage in hypoxic-ischaemic neuronal death. Trends in Neuroscience 18, 58-60.

Choi, D. W., Koh, J. Y., Peters, S., 1988. Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. Journal of Neuroscience 8, 185-196.

Choi, D. W., Maulucci-Gedde, M., Kriegstein, A. R., 1987. Glutamate neurotoxicity in cortical cell culture. Journal of Neuroscience 7, 357-368.

Christie, M. J., James, L. B., Beart, P. M., 1985. An excitant amino acid projection from the medial prefrontal cortex to the anterior part of nucleus accumbens in the rat. Journal of Neurochemistry 45, 477-482.

Christie, M. J., James, L. B., Beart, P. M., 1986. An excitatory amino acid projection from rat prefrontal cortex to periaqueductal gray. Brain Research Bulletin 16, 127-129.

Chung, Y. H., Shin, C., Park, K. H., Cha, C. I., 2000. Immunohistochemical study on the distribution of the voltage-gated calcium alpha-1beta subunit in the mature rat brain. Brain Research 866, 274-280.

Ciccarelli, R., Di Iorio, P., Bruno, V., Battaglia, G., D'Alimonte, I., D'Onofrio, M., Nicoletti, F., Caciagli, F., 1999. Activation of A(1) adenosine or mGlu3 metabotropic glutamate receptors enhances the release of nerve growth factor and S-100beta protein from cultured astrocytes. Glia 27, 275-281.

Ciccarelli, R., Sureda, F. X., Casabona, G., Di Iorio, P., Caruso, A., Spinella, F., Condorelli, D. F., Nicoletti, F., 1997. Opposite influence of the metabotropic glutamate receptor subtypes mGlu3 and -5 on astrocyte proliferation. Glia 21, 390-398.

Ciruela , F., Escriche, M., Burgueño, J., Angulo, E., Casadó, V., Soloviev, M. M., Canela, E. I., Mallol, J., Chan, W-Y., Lluis, C., McIlhinney, R. A. J., Franco, R., 2001. Metabotropic glutamate 1α and adenosine A1 receptors assemble into functionally interacting complexes. Journal of Biological Chemistry 276, 18345-18351.

Ciruela, F., Soloviev, M. M., Chan, W. Y., McIlhinney, R. A., 2000. Homer-1c/Vesl-1L modulates the cell surface targeting of metabotropic glutamate receptor type 1alpha: evidence for an anchoring function. Molecular and Cellular Neurosciences 15, 36-50.

Clarke, G. H., 1990. Developmental cell death: Morphological diversity and multiple mechanisms. Anatomy and Embryology 181, 195-213.

Clifford, D. B., Zorumski, C. F., Olney, J. W., 1989. Ketamine and MK-801 prevent degeneration of thalamic neurons induced by focal cortical seizures. Experimental Neurology 105, 272-279.

Cochilla, A. J., Alford, S., 1998. Metabotropic glutamate receptor-mediated control of neurotransmitter release. Neuron 20, 1007-1016.

Collingridge, G. L., Singer, W., 1990. Excitatory amino acid receptors and synaptic plasticity. Trends in Pharmacological Sciences 11, 290-296.

Colwell, C. S., Levine, M. S., 1996. Excitatory synaptic transmission in neostriatal neurons, regulation by cyclic AMP-dependent mechanisms. Journal of Neuroscience 15, 1704-1713.

Condorelli, D. F., Dell'Albani, P., Amico, C., Lukasiuk, K., Kaczmarek, L., Giuffrida-Stella, A. M., 1994. Glutamate receptor-driven activation of transcription factors in primary neuronal cultures. Neurochemical Research 19, 489-499.

Conn, P. J., Pin, J.-P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annual Review of Pharmacology and Toxicology 37, 205-237.

Constantino, G., Pellicciari, R., 1996. Homology modelling of metabotropic glutamate receptors (mGluRs) structural motifs affecting binding modes and pharmacological profile of mGluR1 agonists and competitive antagonists. Journal of Medicinal Chemistry 39, 3998-4006.

Copani, A., Bruno, V., Battaglia, G., Leanza, G., Pellitteri, R., Russo, A., Stanzani, S., Nicoletti, F., 1995. Activation of metabotropic glutamate receptors protects against apoptosis induced by Beta-amyloid peptide. Molecular Pharmacology 47, 890-897.

Copani, A., Romano, C., Di Giorgi Gerevini, V., Nicosia, A., Casabona, G., Storto, M., Mutel, V., Nicoletti, F., 2000. Reducing conditions differentially affect the functional and structural properties of group-I and -II metabotropic glutamate receptors. Brain Research 867, 165-172.

Cormier, R. J., Mennerick, S., Melbostad, H., Zorumski, C. F., 2001. Basal levels of adenosine modulate mGluR5 on rat hippocampal astrocytes. Glia 33, 24-35.

Corti, C., Restituito, S., Rimland, J. M., Brabet, I., Corsi, M., Pin, J.-P., Ferraguti, F., 1998. Cloning and characterization of alternative mRNA forms for the rat metabotropic glutamate receptors mGluR7 and mGluR8. European Journal of Neuroscience 10, 3629-3641.

Cotman, C. W., Flatman, J. A., Ganong, A. H., Perkins, M. N., 1986. Effects of excitatory amino acid antagonists on evoked and spontaneous excitatory potentials in guinea-pig hippocampus. Journal of Physiology 378, 403-415.

Coyle, J. T., McGeer, E. G., McGeer, P. L., Schwarcz, R., 1978. Neostriatal injections: a model for Huntington's chorea. In, Kainic acid as a tool in neurobiology. Eds: McGeer, E. G., Olney, J. W., McGeer, P. L. Raven Press, New York. pp. 139-160.

Coyle, J. T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689-695.

Coyle, J. T., Zaczek, R., Slevin, J., Collins, J., 1981. Neuronal receptor sites for kainic acid: correlations with neurotoxicity. Advances in Biochemical Psychopharmacology 27, 337-46.

Crepel, V., Anikstejn, L., Ben-Ari, Y., Hammond, C., 1994. Glutamate metabotropic receptors increase a Ca-activated non-specific cationic current in CA1 hippocampal neurons. Journal of Neurophysiology 72, 1561-1569.

Cuesta, M. C., Arcaya, J. L., Cano, G., Sanchez, L., Maixner, W., Suarez-Roca, H., 1999. Opposite modulation of capsaicin-evoked substance P release by glutamate receptors. Neurochemistry International 35, 471-478.

Curtis, D. R., Johnston, G. A. R., 1974. Amino acid transmitters in the mammalian central nervous system. Ergebnisse der Physiologie, Biologischen Chemie und Experimentellen Pharmakologie 69, 97-188.

Curtis, D. R., Watkins, J. C., 1960. The excitation and depression of spinal neurones by structurally related amino acids. Journal of Neurochemistry 6, 117-141.

Dalby, N. O., Thomsen, C., 1996. Modulation of seizure activity in mice by metabotropic glutamate receptor ligands. Journal of Pharmacological and Experimental Therapeutics 276, 516-522.

Danbolt, N. C., 2001. Glutamate uptake. Progress in Neurobiology 65, 1-105.

Daw, M. I., Chittajallu, R., Bortolotto, Z. A., Dev, K. K., Duprat, F., Henley, J. M., Collingridge, G. L., Isaac, J. T., 2000. PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. Neuron 28, 873-886.

Dawson, L., Chadha, A., Megalou, M., Duty, S., 2000. The group II metabotropic glutamate receptor agonist, DCG-IV, alleviates akinesia following intranigral or intraventricular administration in the reserpine-treated rat. British Journal of Pharmacology 129, 541-546.

De Boni, U., McLachlan, D. R., 1985. Controlled induction of paired helical filaments of the Alzheimer type in cultured human neurons, by glutamate and aspartate. Journal of the Neurological Sciences 68, 105-118.

Defer, N., Best-Belpomme, M., Hanoune, J., 2000. Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. American Journal of Physiology and Renal Physiology 279, F400-F416.

Degirolami, U., Crowell, R. M., Marcoux, F. W., 1984. Selective necrosis and total necrosis in focal cerebral ischaemia. Neuropathologic observations on experimental middle cerebral artery occlusion in the macaque monkey. Journal of Neuropathology and Experimental Neurology 43, 57-71.

Desagher, S., Glowinski, J., Premont, J., 1996. Astrocytes protect neurons from hydrogen peroxide toxicity. Journal of Neuroscience 16, 2553-2562.

De Sarro, G., Gitto, R., Rizzo, M., Zappia, M., De Sarro, A., 1996. 1,4-Benzodiazepine derivatives as anticonvulsant agents in DBA/2 mice. General Pharmacology 27, 935-941.

De Sarro, G., Gratteri, S., Bonacci, F., Musumeci, S. A., Elia, M., De Sarro, A., 2000. Topiramate potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. European Journal of Pharmacology 388, 163-170.

Dessi, F., Ben-Ari, Y., Charriaut-Marlangue, C., 1994. Increased synthesis of specific proteins during glutamate-induced neuronal death in cerebellar culture. Brain Research 654, 27-33.

Dessi, F., Charriaut-Marlangue, C., Khrestchatisky, M., Ben-Ari, Y., 1993. Glutamate-induced neuronal death is not a programmed cell death in cerebellar culture. Journal of Neurochemistry 60, 1953-1956.

Dev, K. K., Nakajima, Y., Kitano, J., Braithwaite, S. P., Henley, J. M., Nakanishi, S., 2000. PICK1 interacts with and regulates PKC phosphorylation of mGLUR7. Journal of Neuroscience 20, 7252-7257.

Dev, K. K., Nakanishi, S., Henley, J. M., 2001. Regulation of mglu(7) receptors by proteins that interact with the intracellular C-terminus. Trends in Pharmacological Sciences 22, 355-361.

Dietrich, D., Kral, T., Clusmann, H., Friedl, M., Schramm, J., 1999. Reduced function of L-AP4-sensitive metabotropic glutamate receptors in human epileptic sclerotic hippocampus. European Journal of Neuroscience 11, 1109-1113.

Di Iorio, P., Battaglia, G., Ciccarelli, R., Ballerini, P., Giuliani, P., Poli, A., Nicoletti, F., Caciagli, F., 1996. Interaction between A_1 adenosine and class II metabotropic glutamate receptors in the regulation of purine and glutamate release from rat hippocampal slices. Journal of Neurochemistry 67, 302-309.

D'Mello, S. R., Galli, C., Ciotti, T., Calissano, P., 1993. Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP. Proceedings of the National Academy of Science, United States of America 90, 10989-10993.

Dingledine, R., Borges, K., Bowie, D., Traynelis, S. F., 1999. The glutamate receptor ion channels. Pharmacological Reviews 51, 7-61.

Dingledine, R., McBain, C. J., McNamara, J. O., 1990. Excitatory amino acid receptors in epilepsy. Trends in Pharmacological Sciences 11, 334-338.

Dirnagl, U., Iadecola, C., Moskowitz, M. A., 1999. Pathobiology of ischaemic stroke: an integrated view. Trends in Neurosciences 22, 391-397.

Dobbertin, A., Schmid, P., Gelman, M., Glowinski, J., Mallat, M., 1997. Neurons promote macrophage proliferation by producing transforming growth factor-beta2. Journal of Neuroscience 17, 5305-5315.

D'Onofrio, M., Cuomo, L., Battaglia, G., Ngomba, R. T., Storto, M., Kingston, A. E., Orzi, F., De Blasi, A., Di Iorio, P., Nicoletti, F., Bruno, V., 2001. Neuroprotection mediated by glial group-II metabotropic glutamate receptors requires the activation of the MAP kinase and the phosphatidylinositol-3-kinase pathways. Journal of Neurochemistry 78, 435-445.

Drukarch, B., Schepens, E., Jongenelen, C. A. M., Stoof, J. C., Langeveld, C. H., 1997. Astrocyte-mediated enhancement of neuronal survival is abolished by glutathione deficiency. Brain Research 770, 123-130.

Dudel, J., 1975. Potentiation and desensitization after glutamate induced postsynaptic currents at the crayfish neuromuscular junction. Pflugers Archives - European Journal of Physiology 356, 317-327.

Duggan, A. W., 1974. The differential sensitivity to L-glutamate and L-aspartate of spinal interneurones and Renshaw cells. Experimental Brain Research 19, 522-528.

Dutar, P., Petrozzino, J. J., Vu, H. M., Schmidt, M. F., Perkel, D. J., 2000. Slow synaptic inhibition mediated by metabotropic glutamate receptor activation of GIRK channels. Journal of Neurophysiology 84, 2284-2290.

Dutar, P., Vu, H. M., Perkel, D. J., 1999. Pharmacological characterization of an unusual mGluR-evoked neuronal hyperpolarization mediated by activation of GIRK channels. Neuropharmacology 38, 467-475.

Duvoisin, R.M., Zhang, C., Ramonell, K.A, 1995. A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. Journal of Neuroscience 15, 3075-3083.

East, S. J., Hill, M. P., Brotchie, J. M., 1995. Metabotropic glutamate receptor agonists inhibit endogenous glutamate release from rat striatal synaptosomes. European Journal of Pharmacology 277, 117-21.

Eaton, S. A., Salt, T. E., 1996. Role of N-methyl-D-aspartate and metabotropic glutamate receptors in corticothalamic excitatory postsynaptic potentials in vivo. Neuroscience 73, 1-5.

El Far, O., Airas, J., Wischmeyer, E., Nehring, R. B., Karschin, A., Betz, H., 2000. Interaction of the C-terminal tail region of the metabotropic glutamate receptor 7 with the protein kinase C substrate PICK1. European Journal of Neuroscience 12, 4215-4221.

Emile, L., Mercken, L., Apiou, F., Pradier, L., Bock, M. D., Menager, J., Clot, J., Doble, A., Blanchard, J. C., 1996. Molecular cloning, functional expression, pharmacological characterization and chromosomal localization of the human metabotropic glutamate receptor type 3. Neuropharmacology 35, 523-530.

Eng, D. L., Lee, Y. L., Lal, P. G., 1997. Expression of glutamate uptake transporters after dibutyryl cyclic AMP differentiation and traumatic injury in cultured astrocytes. Brain Research 778, 215-221.

Erecinska, M., Silver, I. A., 1990. Metabolism and role of glutamate in mammalian brain. Progress in Neurobiology 35, 245-296.

Faden, A. I., Demediuk, P., Panter, S. S., Vink, R., 1989. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244, 798-800.

Faden, A. I., Ivanova, S. A., Yakovlev, A. G., Mukhin, A. G., 1997. Neuroprotective effects of group III mGluR in traumatic neuronal injury. Journal of Neurotrauma 14, 885-895.

Faden, A. I., O'Leary, D. M., Fan, L., Bao, W., Mullins, P. G., Movsesyan, V. A., 2001. Selective blockade of the mGluR1 receptor reduces traumatic neuronal injury in vitro and improves outcome after brain trauma. Experimental Neurology 167, 435-444.

Fadok, V. A., 1999. Clearance: the last and often forgotten stage of apoptosis. Journal of Mammary Gland Biology & Neoplasia 4, 203-211.

Fagg, G. E., Foster, A. C., 1983. Amino acid neurotransmitters and their pathways in the mammalian central nervous system. Neuroscience 9, 701-719.

Fagni, L., Chavis, P., Ango, F., Bockaert, J., 2000. Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. Trends in Neuroscience 23, 80-88.

Favaron, M., Manev, H., Simar., R., Bertolino, M., Szekely, A. M., DeErausquin, G., Guidotti, A., Costa, E., 1990. Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. Proceedings of the National Academy of Sciences of the United States of America 87, 1983-1987.

Feldman, D. E., Knudsen, E. I., 1997. NMDA and non-NMDA glutamate receptors in auditory transmission in the barn owl inferior colliculus. Journal of Neuroscience 14, 5939-5958.

Fimia, G. M., Sassone-Corsi, P., 2000. Cyclic AMP signalling. Journal of Cell Science 114, 1971-1972.

Fitzjohn, S. M., Irving, A. J., Palmer, M. J., Harvey, J., Lodge, D., Collingridge, G. L., 1996. Activation of group I mGluRs potentiates NMDA responses in rat hippocampus. Neuroscience Letters 203, 211-213.

Flavin, H. J., Jin, X. T., Daw, N. W., 2000. 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate (APDC) attenuates cortical EPSPs. Brain Research 873, 212-217.

Flor, P. J., Bruno, V., Battaglia, G., Reutlinger, S., Lukic, S., Leonhardt, T., Laurie, D., Sommer, B., Varney, M., Velicelebi, G., Kuhn, R., Urwyler, S., Dreesen, J., Portet, C., Schmutz, M., Nicoletti, F., Gasparini, F., 1998. Neuroprotection in vitro and in vivo by (*R*,*S*)-PPG, a potent and selective group III metabotropic glutamate receptor agonist. Society for Neuroscience Abstracts 24, 1089.

Flor, P. J., Ksiazek, I., Bruno, V., Battaglia, G., Lukic, S., Inderbitzin, W., Van Der Putten, H., Kuhn, R., Nicoletti, F., Hampson, D. R., Gasparini, F., 1999. Reversal of neurodegenerative processes by selective modulation of group-I or group-III metabotropic glutamate receptor subtypes. Neuropharmacology 38, A15-A16.

Flor, P. J., Lindauer, K., Puttner, I., Ruegg, D., Lukic, S., Knopfel, T., Kuhn, R., 1995a. Molecular cloning, functional expression and pharmacological characterization of the human metabotropic glutamate receptor type 2. European Journal of Neuroscience 7, 622-629.

Flor, P. J., Lukic, S., Ruegg, D., Leonhardt, T., Knopfel, T., Kuhn, R., 1995b. Molecular cloning, functional expression and pharmacological characterization of the human metabotropic glutamate receptor type 4. Neuropharmacology 34, 149-155.

Flor, P. J., Gomeza, J., Tones, M. A., Kuhn, R., Pin, J.-P., Knophel, T., 1996. The C-terminal domain of the mGluR1 metabotropic glutamate receptor affects sensitivity to agonists. Journal of Neurochemistry 67, 58-63.

Flor, P. J., Van Der Putten, H., Ruegg, D., Lukic, S., Leonhardt, T., Bence, M., Sansig, G., Knopfel, T., Kuhn, R., 1997. A novel splice variant of a metabotropic glutamte receptor, human mGluR7b. Neuropharmacology 36, 153-159.

Fonnum, F., 1984. Glutamate: a neurotransmitter in mammalian brain. Journal of Neurochemistry 42, 1-11.

Fonnum, F., 1991. Neurochemical studies on glutamate-mediated neurotransmission. In, Excitatory Amino Acids (Vol.5). Eds: Meldrum, B. S., Moroni, F., Simon, R. P., Woods, J. H. Raven Press, New York. pp. 15-25

Fonnum, F., Storm-Mathisen, J., Divac, I., 1981. Biochemical evidence for glutamate as neurotransmitter in corticostriatal and corticothalamic fibres in rat brain. Neuroscience 6, 863-873.

Ford, C. E., Skiba, N. P., Bae, H, Daaka, Y., Reuveny, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C-S., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., Hamm, H. E., 1998. Molecular basis for interactions of G protein βγ subunits with effectors. Science 280, 1271-1274.

Forloni, G., Grzanna, R., Blakely, R. D., Coyle, J. T., 1987. Co-localization of N-acetyl-aspartyl-glutamate in central cholinergic, noradrenergic, and serotonergic neurons. Synapse 1, 455-460.

Forsythe, I. D., Clements, J. D., 1990. Presynaptic glutamate receptors depress excitatory monosynaptic transmission between mouse hippocampal neurones. Journal of Physiology 429, 1-16.

Fotuhi, M., Standaert, D. G., Testa, C. M., Penny Jr., J. B., Young, A. B., 1994. Differential expression of metabotropic glutamate receptors in the hippocampus and entorhinal cortex of the rat. Molecular Brain Research 21, 283-292.

Frandsen, A., Drejer, J., Schousboe, A., 1989. Direct evidence that excitotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. Journal of Neurochemistry 53, 297-299.

Frandsen, A., Schousboe, A., 1990. Development of excitatory amino acid induced cytotoxicity in cultured neurons. International Journal of Developmental Neuroscience 8, 209-216.

Franklin, K., Paxinos, G., 1997. The mouse brain in stereotaxic coordinates. Academic Press, San Diego.

Gahwiler, B. H., Capogna, M., Debanne, D., McKinney, R. A., Thompson, S. M., 1997. Organotypic slice cultures: a technique has come of age. Trends in Neurosciences 20, 471-477.

Garcia-Ladona, F. J., Palacios, J. M., Probst, A., Wieser, H. G., Mengod, G., 1994. Excitatory amino acid AMPA receptor mRNA localization in several regions of normal and neurological disease affected human brain. An in situ hybridization histochemistry study. Molecular Brain Research 21, 75-84.

Gasparini, F., Bruno, V., Battaglia, G., Lukic, S., Leonhardt, T., Inderbitzin, W., Laurie, D., Sommer, B., Varney, M. A., Hess, S. D., Johnson, E. C., Kuhn, R., Urwyler, S., Sauer, D., Portet, C., Schmutz, M., Nicoletti, F., Flor, P. J., 1999. (R,S)-4-phosphonophenylglycine, a potent and selective group III metabotropic glutamate receptor agonist, is anticonvulsive and neuroprotective in vivo. Journal of Pharmacology and Experimental Therapeutics 289, 1678-1687

Genazzani, A. A., Casabona, G., L'Episcopo, M. R., Condorelli, D. F., Dell'Albani, P., Shinozaki, H., Nicoletti, F., 1993. Characterization of metabotropic glutamate receptors negatively linked to adenylyl cyclase in brain slices. Brain Research 622, 132-138.

Genazzani, A. A., L'Episcopo, M. R., Casabona, G., Shinozaki, H., Nicoletti, F., 1994. (2*S*, 1'*R*,2'*R*, 3'*R*)-2-(2,3-Dicarbxycyclopropyl)glycine positively modulates metabotropic glutamate receptors coupled to polyphosphoinositide hydrolysis in rat hippocampal slices. Brain Research 659, 10-16.

Gereau IV, R. W., Conn, J. P., 1995. Roles of specific metabotropic glutamate receptor subtypes in regulation of hippocampal CA1 pyramidal cell excitability. Journal of Neurophysiology 74, 122-129.

Ghauri, M., Chapman, A., Meldrum, B. S., 1996. Convulsant and anticonvulsant actions of agonists and antagonists of group III mGluRs. NeuroReport 7, 1469-1474.

Giardina, S. F., Cheung, N. S., Reid, M. T., Beart, P. M., 1998. Kainate-induced apoptosis in cultured murine cerebellar granule cells elevates expression of the cell cycle gene cyclin D1. Journal of Neurochemistry 71, 1325-1328.

Gill, R., Lodge, D., 1997. Pharmacology of AMPA anatgonists and their role in neuroprotection. In, Neuroprotective agents and cerebral ischaemia. Eds: Green, A. R., Cross, A. J. Academic Press, New York. pp. 197-232.

Gillardon, F., Wickert, H., Zinmerman, M., 1995. Up-regulation of bax and down-regulation of bcl-2 is associated with kainate-induced apoptosis in mouse brain. Neuroscience Letters 192, 85-88.

Glazier, S., Bell, W., Nosel, E., Pons, T., Tytell, M., Deadwyler, S., Grigorenko, E. V., 1997. Changes in glutamate receptor subunit composition in hippocampal and cortex patients with refractory epilepsy. Epilepsia 38 (Suppl 3), 191.

Goldberg, M. P., Strasser, U., Dugan, L. L., 1997. Techniques for assessing neuroprotective drugs in vitro. In, Neuroprotective agents and cerebral ischaemia. Eds, Green, A.R., Cross, A.J. Academic Press, London. pp. 47-69.

Gomeza, J., Joly, C., Kuhn, R., Knophel, T., Bockeart, J., Pin, J.-P., 1996b. The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins. Journal of Biological Chemistry 271, 2199-2205.

Gomeza, J., Mary, S., Brabet, I., Parmentier, M.-L., Restituito, S., Bockaert, J., Pin, J.-P., 1996a. Coupling of metabotropic glutamate receptors 2 and 4 to Ga15, Ga16, and chimeric Gaq/i proteins: characterization of new antagonists. Molecular Pharmacology 50, 923-930.

Gower, A. J., Hirsch, E., Boehrer, A., Noyer, M., Marescaux, C., 1992. Effects of levetiracetam, a novel antiepileptic drug, on convulsant activity in two genetic rat models of epilepsy. Epilepsy Research 22, 207-213.

Gray, C. W., Patel, A. J., 1995. Neurodegeneration mediated by glutamate and beta-amyloid peptide: a comparison and possible interaction. Brain Research 691, 169-179.

Han, G., Hampson, D. R., 1999. Ligand binding of the amino-terminal domain of the mGluR4 subtype of metabotropic glutamate receptor. Journal of Biological Chemistry 274, 10008-10013.

Hanania, T., Johnson, K. M., 1999. Regulation of NMDA-stimulated [14C]GABA and [3H]acetylcholine release by striatal glutamate and dopamine receptors. Brain Research 844, 106-117.

Hansson, O., Peters N. A., Leist, M., Nicotera, P., Castilho, R. F., Brundin, P., 1999. Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. Proceedings of the National Academy of Sciences of the United States of America 96, 8727-8732.

Hashimoto, T., Inazawa, J., Okamoto, N., Tagawa, Y., Bessho, Y., Honda, Y., Nakanishi, S., 1997. The whole nucleotide sequence and chromosomal localization of the gene for human metabotropic glutamate receptor subtype 6. European Journal of Neuroscience 9, 1226-1235.

Hattori, A., Katayama, M., Iwasaki, S., Ishii, K., Tsujimoto, M., Kohno, M., 1999. Bone morphogenetic protein-2 promotes survival and differentiation of striatal GABAergic neurons in the absence of glial cell proliferation. Journal of Neurochemistry 72, 2264-2271.

Hayashi, T., 1954. Effects of sodium glutamate on the nervous system. Keio Journal of Medicine 3, 183-192.

Hayashi, Y., Moriyama, A., Takahashi, T., Ohishi, H., Ogawa-Meguro, R., Shigemoto, R., Mizuno, N., Nakanishi, S., 1993. Role of metabotropic glutamate receptor in synaptic modulation in the accessory olfactory bulb. Nature 366, 687-690.

Hayashi, Y., Tanabe, Y., Aramori, I., Masu, M., Shimamoto, K., Ohfune, Y., Nakanishi, S., 1992. Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. British Journal of Pharmacology 107, 539-543.

Hayashi, Y., Sekiyama, N., Nakanishi, S., Jane, D. E., Sunter, D. C., Birse, E. F., Udvarhelyi, P. M., Watkins, J. C., 1994. Analysis of agonist and antagonist activities of phenylglycine derivatives for different cloned metabotropic glutamate receptor sub-types. Journal of Neuroscience 14, 3370-3377.

Hearn, T. J., Ganong, A. H., Cotman, C. W., 1986. Antagonism of lateral olfactory tract synaptic potentials in rat prepyriform cortex slices. Brain Research 379, 372-376.

Heck, S., Enz, R., Richter-Landsberg, C., Blohm, D. H., 1997. Expression of eight metabotropic glutamate receptor subtypes during neuronal differentiation of P19 embryocarcinoma cells: a study by RT-PCR and in situ hybridization. Developmental Brain Research 101, 85-91.

Henrich-Noack, P., Flor, P. J., Sabelhaus, C. F., Prass, K., Dirnagl, U., Gasparini, F., Sauter, A., Rudin, M., Reymann, K. G., 2000. Distinct influence of the group III metabotropic glutamate receptor agonist (R,S)-4-phosphonophenylglycine [(R,S)-PPG] on different forms of neuronal damage. Neuropharmacology 39, 911-917.

Henrich-Noack, P., Prass, K., Dirnagl, U., Sabelhaus, C. F., Reymann, K. G., 1999. Effects of R,S-PPG, a potent and selective mGluR III agonist, in models of excitotoxicity and ischemia. Neuropharmacology 38, A18-19.

Hermans, E., Nahorski, S. R., Challis, R. A., 1998. Reversible and non-competitive antagonist profile of CPCCOEt at the human type 1alpha metabotropic glutamate receptor. Neuropharmacology 37, 1645-1647.

Herrero, I., Castro, E., Miras-Portugal, M. T., Sanchez-Prieto, J., 1996. Two components of glutamate exocytosis differentially affected by presynaptic modulation. Journal of Neurochemistry 67, 2346-2354.

Herrero, I., Miras-Portugal, M. T., Sanchez-Prieto, J., 1992. Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. Nature 360, 163-166.

Herrero, I., Miras-Portugal, M. T., Sanchez-Prieto, J., 1998. Functional switch from facilitation to inhibition in the control of glutamate release by metabotropic glutamate receptors. Journal of Biological Chemistry 273, 1951-1958.

Hosford, D. A., Clark, S. Cao, Z. Wilson, W. A., Lin, F., Morrisett, R. A., Huin, A., 1992. The role of $GABA_B$ receptor activation in absence seizures of lethargic (lh/lh) mice. Science 257, 398-401.

Hoshi, T., Garber, S.S., Aldrich, R.W., 1988. Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation. Science 240, 1652-1655.

Hu, G., Duffy, P., Swanson, C., Ghasemzadeh, M. B., Kalivas, P. W., 1999. The regulation of dopamine transmission by metabotropic glutamate receptors. Journal of Pharmacology and Experimental Therapeutics 289, 412-416.

Huang, L., Rowan, M. J., Anwyl, R., 1999. Induction of long-lasting depression by (+)-alpha-methyl-4-carboxyphenylglycine and other group II mGlu receptor ligands in the dentate gyrus of the hippocampus in vitro. European Journal of Pharmacology 366, 151-158.

Ikeuchi, T., Shimoke, K., Kubo, T., Yamada, M., Hatanaka, H., 1998. Apoptosis-inducing and -preventing signal transduction pathways in cultured cerebellar granule neurons. Human Cell 11, 125-140.

Ikonomidou, C., Turski, L., 1995. Excitotoxicity and neurodegenerative diseases. Current Opinion in Neurology 8, 487-497.

Irving, A. J., Schofield, J. G., Watkins, J. C., 1990. 1*S*,3*R*-ACPD stimulates and L-AP3 blocks Ca^{2+} mobilization in rat cerebellar neurons. European Journal of Pharmacology 186, 363-365.

Ishida, M., Saitoh, T., Shimamoto, K., Ohfune, Y., Shinozaki, H., 1993b. A novel metabotropic glutamate receptor agonist: marked depression of monosynaptic excitation in the isolated newborn rat spinal cord. British Journal of Pharmacology 109, 1169-117.

Ishida, M., Saitoh, T., Shinozaki, H., 1993a. A new metabotropic glutamate receptor agonist: developmental change of its sensitivity to receptors in the newborn rat spinal cord. Neuroscience Letters 160, 156-158.

Ito, I., Kohda, A., Tanabe, S., Hirose, E., Hayashi, M., Mitsunaga, S., Sugiyama, H., 1992. 3,5-Dihydroxyphenylglycine: a potent agonist of metabotropic glutamate receptors. NeuroReport 3, 1013-1016.

Iqbal, K., Alonso, A. D., Gondal, J. A., Gong, C. X., Haque, N., Khatoon, S., Sengupta, A., Wang, J. Z., Grundke-Iqbal, I., 2000. Mechanism of neurofibrillary degeneration and pharmacologic therapeutic approach. Journal of Neural Transmission Supplementum 59, 213-222.

Jacobowitz, O., Chen, J., Premont, R. T., Iyengar, R. 1993. Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. Journal of Biological Chemistry 268, 3829-3832.

Jane, D. E., Jones, P. L. S. J., Pook, P. C.-K., Tse, H.-W., Watkins, J. C., 1994. Actions of two new antagonists showing selectivity for different sub-types of metabotropic glutamate receptor in the neonatal rat spinal cord. British Journal of Pharmacology 112, 809-816.

Jane, D. E., Thomas, N. K., Tse, H. W., Watkins, J. C., 1996. Potent antagonists at the L-AP4- and (1S,3S)-ACPD-sensitive presynaptic metabotropic glutamate receptors in the neonatal rat spinal cord. Neuropharmacology 35, 1029-1035.

Janaky, R., Varga, V., Saransaari, P., Oja, S. S., 1994. Glutamate agonists and [3H]GABA release from rat hippocampal slices: involvement of metabotropic glutamate receptors in the quisqualate-evoked release. Neurochemical Research 19, 729-734.

Janssens, N., Lesage, A. S. J., 2001. Glutamate receptor subunit expression in primary neuronal and secondary glial cultures. Journal of Neurochemistry 77, 1457-1474.

Jeffery, G., Sharp, C., Malitschek, B., Salt, T. E., Kuhn, R., Knopfel, T., 1996. Cellular localisation of metabotropic glutamate receptors in the mammalian optic nerve: a mechanism for axon-glia communication. Brain Research 741, 75-81.

Jellinger, K. A., 1998. The neuropathological diagnosis of Alzheimer disease. Journal of Neural Transmission Supplementum 53, 97-118.

Jia, Z. P., Lu, Y. M., Henderson, J. T., Taverna, F., Romano, C., Abramow-Newerly, W., Wojtowicz, J. M., Roder, J. C., 1998. Selective abolition of the NMDA component of long-term potentiation in mice lacking mGluR5. Learning Memory 5, 331-343.

Jobe, P. C., Picchioni, A. L., Chin, L., 1973. Role of brain norepinephrine in audiogenic seizure in the rat. Journal of Pharmacology and Experimental Therapeutics 184, 1-10.

Joels, M., Van Veldhuizen, M., Urban, I. J., De Kloet, E. R., 1987. N-acetyl-aspartylglutamate: binding sites and excitatory action in the dorsolateral septum of rats. Brain Research 403, 192-197.

Johansen, P. A., Robinson, M. B., 1995. Identification of 2-amino-2-methyl-4-phosphonobutanoic acid as an antagonist at the $mGlu_{4a}$ receptor. European Journal of Pharmacology 290, R1-R3.

John, C. A., Beart, P. M., Giardina, S. F., Pascoe, C. J., Cheung, N. S., 1999. Cyclothiazide and GYKI 52466 modulate AMPA receptor-mediated apoptosis in cortical neuronal cultures. Neuroscience Letters 268, 9-12.

Johnson, B. G., Wright, R. A., Arnold, M. B., Wheeler, W. J., Ornstein, P. J., Schoepp, D. D., 1999. [^{3}H]-LY341495 as a novel rapid filtration antagonist radioligand for group II metabotropic glutamate receptors: Characterization of binding to membranes of mGlu receptor subtype expressing cells. Neuropharmacology 38, 1519-1529.

Johnston, G. A. R., 1973. Convulsions induced in 10-day-old rats by intraperitional injection of monosodium glutamate and related excitant amino acids. Biochemical Pharmacology 22, 137-140.

Johnston, G. A. R., Kennedy, S. M. F., Twitchin, B., 1979. Action of the neurotoxin kainic acid on high affinity uptake of L-glutamic acid in rat brain slices. Journal of Neurochemistry 32, 121-127.

Jones, N. M., Beart, P. M., Monn, J. A., Widdop, R. E., 1999. Type I and II metabotropic glutamate receptors mediate depressor and bradycardic actions in the nucleus of the solitary tract of anaesthetized rats. European Journal of Pharmacology 380, 129-135.

Jones, N. M., Monn, J. A., Beart, P. M., 1998. Type I and II metabotropic glutamate receptors regulate the outflow of [3H]D-aspartate and [14C]gamma-aminobutyric acid in rat solitary nucleus. European Journal of Pharmacology 353, 43-51.

Jones, P. G., Roberts, P. J., 1990. Ibotenate stimulates glutamate release from guinea pig cerebrocortical synaptosomes: inhibition by L-2-amino-4-phosphonobutyrate (L-AP4). Neuroscience Letters 111, 228-232.

Jouvenceau, A., Dutar, P., Billard, J. M., 1995. Presynaptic depression of inhibitory postsynaptic potentials by metabotropic glutamate receptors in rat hippocampal CA1 pyramidal cells. European Journal of Pharmacology 281, 131-139.

Jurkowitz, M. S., Litsky, M. L., Browning, M. J., Hohl, C. M., 1998. Adenosine, inosine, and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation. Journal of Neurochemistry 71, 535-548.

Kaatz, K. W., Albin, R. L., 1995. Intrastriatal and intrasubthalamic stimulation of metabotropic glutamate receptors: a behavioral and Fos immunohistochemical study. Neuroscience 66, 55-65.

Kamiya, H., Yamamoto, C., 1997. Phorbol ester and forskolin suppress the presynaptic inhibitory action of group-II metabotropic glutamate receptor at rat hippocampal mossy fibre synapse. Neuroscience 80, 89-94.

Kanai, Y., Trotti , D., Nussberger, S., Hediger, M. A., 1997. The high affinity glutamate transporter family. In Neurotranmitter Transporters: Structure, function and regulation. Ed. Reith, M. E. A. Humana Press Inc. Totowa. pp.467

Kastner, A., Hirsch, E. C., Agid, Y., Javoy-Agid, F., 1993. Tyrosine hydroxylase protein and messenger RNA in the dopaminergic nigral neurons of patients with Parkinson's disease. Brain Research 606, 341-345.

Kato, A., Ozawa, F., Saitoh, Y., Fukazawa, Y., Sugiyama, H., Inokuchi, K., 1998. Novel members of the Ves1/Homer family of PDZ proteins that bind metabotropic glutamate receptors. Journal of Biological Chemistry 273, 23969-23975.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., Graybiel, A. M., 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282, 2275-2279.

Kemp, M. C., Jane, D. E., Tse, H.-W., Roberts, P. J., 1996. Agonists of cyclic AMP-coupled metabotropic glutamate receptors in adult-rat cortical slices. European Journal of Pharmacology 309, 79-85.

Kemp, M. C., Roberts, P. J., Pook, P. C.-K., Jane, D. E., Jones, A. W., Jones, P. L. S.-J., Sunter, D. C., Udvarhelyi, P. M., Watkins, J. C., 1994. Antagonism of presynaptically mediated depressant

responses and cyclic AMP-coupled metabotropic glutamate receptors. European Journal of Pharmacology 266, 187-192.

Kennedy, M. B., 1998. Signal transduction molecules at the glutamatergic postsynaptic membrane. Brain Research Reviews 26, 243-257.

Kerner, J. A., Standaert, D. G., Penny Jr., J. B., Young, A. B., Landwehrmeyer, G. B., 1997. Expression of group one metabotropic glutamate receptor subunit mRNAs in neurochemically identified neurons in the rat neostriatum, neocortex and hippocampus. Molecular Brain Research 48, 259-269.

Kerr, J. F. R., 1995. Anatomical methods in cell death. In, Cell Death. Eds: Schwartz, L. M., Osborne, B. A. Academic Press, New York. pp. 1-28.

Khurgel, M., Switzer, R. C. r., Teskey, G. C., Spiller, A. E., Racine, R. J., Ivy, G. O., 1995. Activation of astrocytes during epileptogenesis in the absence of neuronal degeneration. Neurobiology of Disease 2, 23-35.

Kilbride, J., Huang, L. Q., Rowan, M. J., Anwyl, R., 1998. Presynaptic inhibitory action of the group II metabotropic glutamate receptor agonists, LY354740 and DCG-IV. European Journal of Pharmacology 356, 149-157.

Kimura, H., Okamoto, K., Sakai, Y., 1985a. Pharmacological characterization of postsynaptic receptors for excitatory amino acids in Purkinje cell dendrites in the guinea pig cerebellum. Journal of Pharmacobio-Dynamics 8, 119-27.

Kimura, H., Okamoto, K., Sakai, Y., 1985b. Pharmacological evidence for L-aspartate as the neurotransmitter of cerebellar climbing fibres in the guinea-pig. Journal of Physiology 365, 103-119.

Kingston, A. E., O'Neill, M. J., Lam, A., Bales, K. R., Monn, J. A., Schoepp, D. D., 1999. Neuroprotection by metabotropic glutamate receptor agonists: LY354740, LY379268 and LY389795. European Journal of Pharmacology 377, 155-165.

Kingston, A. E., Ornstein, P. L., Wright, R. A., Johnson, B. G., Mayne, N. G., Burnett, J. P., Belagaje, R., Wu, S., Schoepp, D. D., 1998. LY341495 is a nanomolar potent and selective antagonist of group II metabotropic glutamate receptors. Neuropharmacology 37, 1-12.

Kinoshita, A., Ohishi, H., Nomura, S., Shigemoto, R., Nakanishi, S., Mizuno, N., 1996. Presynaptic localization of a metabotropic glutamate receptor, mGluR4a, in the cerebellar cortex: a light and electron microscope study in the rat. Neuroscience Letters 207, 199-202.

Kinoshita, A., Shigemoto, R., Ohishi, H., Van Der Putten, H., Mizuno, N., 1998. Immunohistochemical localization of metabotropic glutamate receptors, mGlu7a and mGlu7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. Journal of Comparative Neurology 393, 332-352.

Kitano, J., Kimura, K., Yamazaki, Y., Soda, T., Shigemoto, R., Nakajima, Y., Nakanishi, S., 2002. Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. Journal of Neuroscience 22, 1280-1289.

Klein, J., Iovino, M., Vakil, M., Shinozaki, H., Loffelholz, K., 1997a. Ontogenetic and pharmacological studies on metabotropic glutamate receptors coupled to phospholipase D activation. Neuropharmacology 36, 305-311.

Klein, J., Reymann, K.G., Riedel, G., 1997b. Activation of phospholipase C and D by the novel metabotropic glutamate receptor agonist tADA. Neuropharmacology 36, 261-263.

Klitgaard, H., Laudrup, P., 1993. Metabotropic excitatory amino acid receptor agonists selectively potentiate behavioural effects induced by ionotropic excitatory amino acids in mice. European Journal of Pharmacology 250, 9-13.

Klockgether, T., Turski, L., Honore, T., Zhang, Z. M., Gash, D. M., Kurlan, R., Greenamyre, J. T., 1991. The AMPA receptor antagonist NBQX has antiparkinsonian effects in monoamine-depleted rats and MPTP-treated monkeys. Annals of Neurology 30, 717-723.

Klodzinska, A., Bijak, M., Chojnacka-Wojcik, E., Kroczka, B., Swaider, M., Czuczwar, S. J., Pilc, A., 2000. Roles of group II metabotropic glutamate receptors in modulation of seizure activity. Naunyn-Schmiedeberg's Archives of Pharmacology 361, 283-288.

Knoflach, F., Kemp, J. A., 1998. Metabotropic glutamate group II receptors activate a G protein-coupled inwardly rectifying K+ current in neurones of the rat cerebellum. Journal of Physiology 509, 347-354.

Knopfel, T., Lukic, S., Leonardt, T., Flor, P. J., Kuhn, R., Gasparini, F., 1995. Pharmacological characterization of MCCG and MAP4 at the mGluR1b, mGluR2 and mGluR4a human metabotropic glutamate receptor subtypes. Neuropharmacology 34, 1099-1102.

Koh, J. Y., Choi, D. W., 1991. Selective blockade of non-NMDA receptors does not block rapidly triggered glutamate-induced neuronal death. Brain Research 548, 318-321.

Koller, K. J., Zaczek, R., Coyle, J. T., 1984. N-acetyl-aspartyl-glutamate: regional levels in rat brain and the effects of brain lesions as determined by a new HPLC method. Journal of Neurochemistry 43, 1136-1142.

Koulen, P., Brandstatter, J. H., 2002. Pre- and Postsynaptic Sites of Action of mGluR8a in the mammalian retina. Investigative Ophthalmology and Visual Science 43, 1933-1940.

Koulen, P., Kuhn, R., Wassle, H., Brandstatter, J. H., 1999. Modulation of the intracellular calcium concentration in photoreceptor terminals by a presynaptic metabotropic glutamate receptor. Proceedings of the National Academy of Sciences of the United States of America 96, 9909-9914.

Kowal, D. M., Hsiao, C., Wardell-Swanson, J., Tasse, J. R., 1998. Correlation between mGluR ligand activities observed in CHO cells transfected with hmGluR4a using [^{3}H]L-AP4 and [^{35}S]GTP[gamma]S binding assays. Society for Neuroscience Abstracts 24, 576.

Kozikowski, A. P., Araldi, G. L., Tuckmantel, W., Pshenichkin, S., Surina, E., Wroblewski, J. T., 1999. 1-amino-APDC, a partial agonist of group II metabotropic glutamate receptors with neuroprotective properties. Bioorganic and Medicinal Chemistry Letters 9, 1721-1726.

Kozikowski, A. P., Steensma, D., Luca-Araldi, G., Tuckmantel, W., Wang, S., Pschenichkin, S., Surina, E., Wroblewski, J. T., 1998. Synthesis and biology of the conformationally restricted ACPD analog, 2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid-I, a potent mGluR agonist. Journal of Medicinal Chemistry 41, 1641-1650.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J., Mignotte, B., 1995. The biochemistry of programmed cell death. FASEB Journal 9, 1277-1286.

Kwak, S., Miyamoto, M., Ishida, M., Shinozaki, H., 1996. Neurotoxicity of (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine, a potent agonist for class II metabotropic glutamate receptors, in the rat. Neuroscience 73, 687-695.

Lafon-Cazal, M., Fagni, L., Guiraud, M.-J., Mary, S., Lerner-Natoli, M., Pin, J.-P., Shigemoto, R., Bockaert, J., 1999b. mGluR7-like metabotropic glutamate receptors inhibit NMDA-mediated excitotoxicity in cultured mouse cerebellar granule neurons. European Journal of Neuroscience 11, 663-672.

Lafon-Cazal, M., Viennois, G., Kuhn, R., Malitschek, B., Pin, J.-P., Shigemoto, R., Bockaert, J., 1999a. mGluR7-like receptor and GABA(B) receptor activation enhance neurotoxic effects of N-methyl-D-aspartate in cultured mouse striatal GABAergic neurones. Neuropharmacology 38, 1631-1640.

Lam, A. G. M., Monn, J. A., Schoepp, D. D., Lodge, D., McCulloch, J., 1999. Group II selective metabotropic glutamate receptor agonists and local cerebral glucose use in the rat. Journal of Cerebral Blood Flow and Metabolism 19, 1083-1091.

Lam, A. G. M., Soriano, M. A., Monn, J. A., Schoepp, D. D., Lodge, D., McCulloch, J., 1998. Effects of the selective metabotropic glutamate agonist LY354740 in a rat model of permanent ischaemia. Neuroscience Letters 254, 121-123.

Lang, C. H., Ajmal, M., 1995. Metabolic, hormonal, and hemodynamic changes induced by metabotropic excitatory amino acid agonist (1*S*,3*R*)-ACPD. American Journal of Physiology 268, R1026-R1033.

Lann, J. A., Cheung, N. S., Beart, P. M., 1997. Apoptosis induced via AMPA-selective glutamate receptors in cultured murine cortical neurons. Journal of Neurochemistry 69, 617-622.

Laurie, D. J., Schoeffter, P., Wiederhold, K. H., Sommer, B., 1997. Cloning, distribution and functional expression of the human $mGlu_6$ metabotropic glutamate receptor. Neuropharmacology 36, 145-152.

Lea, P. M., Wroblewska, B., Sarvey, J. M., Neale, J. H., 2001. Beta-NAAG rescues LTP from blockade by NAAG in rat dentate gyrus via the type 3 metabotropic glutamate receptor. Journal of Neurophysiology 85, 1097-1106.

Lee, R. K., Araki, W., Wurtman, R. J., 1997. Stimulation of amyloid precursor protein synthesis by adrenergic receptors coupled to cAMP formation. Proceedings of the National Academy of Sciences of the United States of America 94, 5422-5426.

Lee, R. K., Wurtman, R. J., 1997. Metabotropic glutamate receptors increase amyloid precursor protein processing in astrocytes: inhibition by cyclic AMP. Journal of Neurochemistry 68, 1830-1835.

Lee, R. K., Wurtman, R. J., Cox, A. J., Nitsch, R. M., 1995. Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America 92, 8083-8087.

Le Gal La Salle, G., 1988. Long-lasting and sequential increase of c-fos oncoprotein expression in kainic acid-induced status epilepticus Neuroscience Letters 88, 127-130.

Leist, M., Nicotera, P., 1998. Apoptosis, excitotoxicity, and neuropathology. Experimental Cell Research 239, 183-201.

Li, X. C., Beart, P. M., Monn, J. A., Jones, N. M., Widdop, R. E., 1999. Type I and II metabotropic glutamate receptor agonists and antagonists evoke cardiovascular effects after intrathecal administration in conscious rats. British Journal of Pharmacology 128, 823-829.

Lie, A. A., Becker, A., Behle, K., Beck, H., Malitschek, B., Conn, J. P., Kuhn, R., Nitsch, R., Plaschke, M., Schramm, J., Elger, C. E., Wiestler, O. D., Blumcke, I., 2000. Upregulation of the metabotropic glutamate receptor mGluR4 in hippocampal neurons with reduced seizure vulnerability. Annals of Neurology 47, 26-35.

Lim, S., Naisbitt, S., Yoon, J., Hwang, J. I., Suh, P. G., Sheng, M., Kim, E., 1999. Characterization of the Shank family of synaptic proteins. Multiple genes, alternative splicing, and differential expression in brain and development. Journal of Biological Chemistry 274, 29510-29518.

Lin, C. L., Bristol, L. A., Jin, L., Dykes-Hoberg, M., Crawford, T., Clawson, L., Rothstein, J. D., 1998. Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron 20, 589-602.

Lin, F., Barun, S., Lutz, C. M., Wang, Y., Hosford, D. A., 1999. Decreased $^{45}Ca^{2+}$ uptake in P/Q-type calcium channels in homozygous lethargic (*Cacnb4^{lh}*) mice is associated with increased beta-3 and decreased beta-4 calcium subunit mRNA expression. Molecular Brain Research 71, 1-10.

Lipton, S. A., Rosenberg, P. A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. New England Journal of Medicine 330, 613-622.

Litchfield, J. T., Wilcoxon, F., 1949. A simplified method of evaluating dose effect experiments. Journal of Pharmacology and Experimental Therapeutics 96, 99-113.

Litschig, S., Gasparini, F., Rueegg, D., Stoehr, N., Flor, P. J., Vranesic, I., Prezeau, L., Pin, J.-P., Thomsen, C., Kuhn, R., 1999. CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. Molecular Pharmacology 55, 453-461.

Lombardi, G., Alesiani, M., Leonardi, P., Cherici, G., Pellicciari, R., Moroni, F., 1993. Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[3H]-aspartate output in rat striatum. British Journal of Pharmacology 110, 1407-1412.

Loscher, W., 1998. Pharmacology of glutamate receptor antagonists in the kindling model of epilepsy. Progress in Neurobiology 54, 721-741.

Lowenstein, D. H., 1996. Recent advances related to basic mechanisms of epileptogenesis. Epilepsy Research - Supplement 11, 45-60.

Lowenstein, D. H., Thomas, M. J., Smith, D. H., McIntosh, T. K., 1992. Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. Journal of Neuroscience 12, 4846-4853.

Lu, Y. M., Jia, Z. P., Janus, C., Henderson, J. T., Gerlai, R., Wojtowicz, J. M., 1997. Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. Journal of Neuroscience 17, 5196-5205.

Lucas, D. R., Newhouse, J. P., 1957. The toxic effect of sodium L-glutamate on the inner layers of the retina. Archives in Opthalmology 58, 193-201.

Lui, F.-C., Wu, G.-C., Hsieh, S.-T., Lai, H.-L., Wang, H.-F., Wang, T.-W., Chern, Y., 1998. Expression of type VI adenylyl cyclase in the central nervous system: implication for a potential regulator of multiple signals in different neurotransmitter systems. FEBS Letters 436, 92-98.

Lujan, R., Nusser, Z., Roberts, D. B., Shigemoto, R., Somogyi, P., 1996. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendrites and dendritic spines in the rat hippocampus. European Journal of Neuroscience 8, 1488-1500.

Lujan, R., Roberts, J. D., Shigemoto, R., Ohishi, H., Somogyi, P., 1997. Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. Journal of Chemical Neuroanatomy 13, 219-241.

Luscher, C., Jan, L. Y., Stoffel, M., Malenka, R. C., Nicoll, R. A., 1997. G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19, 687-695.

Maccaferri, G., Toth, K., McBain, C. J., 1998. Target-specific expression of presynaptic mossy fiber plasticity. Science 279, 1368-1370.

Macek, T. A., Winder, D. G., Gereau, R. W., Ladd, C. O., Conn, P. J., 1996. Differential involvement of group II and group III mGluRs as autoreceptors at lateral and medial perforant path synapses. Journal of Neurophysiology 76, 3798-3806.

Maginn, M., Caldwell, M., Kelly, J. P., Leonard, B. E., 1995. The effect of 2-amino-3-phosphonopropionic acid (AP-3) in the gerbil model of cerebral ischaemia. European Journal of Pharmacology 282, 259-262.

Maione, S., Palazzo, E., de Novellis, V., Stella, L., Leyva, J., Rossi, F., 1998. Metabotropic glutamate receptors modulate serotonin release in the rat periaqueductal gray matter. Naunyn-Schmiedebergs Archives of Pharmacology 358, 411-417.

Makoff, A., Lelchuk, R., Oxer, M., Harrington, K., Emson, P., 1996b. Molecular characterization and localization of human metabotropic glutamate receptor type 4. Molecular Brain Research 37, 239-248.

Makoff, A., Phillips, T., Pilling, C., Emson, P., 1997. Expression of a novel splice variant of human mGluR1 in the cerebellum. NeuroReport 8, 2943-2947.

Makoff, A., Pilling, C., Harrington, K., Emson, P., 1996c. Human metabotropic glutamate receptor type 7: molecular cloning and mRNA distribution in the CNS. Molecular Brain Research 40, 165-170.

Makoff, A., Volpe, F., Lelchuk, R., Harrington, K., Emson, P., 1996a. Molecular characterization and localization of human metabotropic glutamate receptor type 3. Molecular Brain Research 40, 55-63.

Malcolm, C. S., Ritchie, L., Grieve, A., Griffiths, R., 1996. A prototypic intracellular calcium antagonist, TMB-8, protects cultured cerebellar granule cells against the delayed, calcium-dependent component of glutamate neurotoxicity. Journal of Neurochemistry 66, 2350-2360.

Malherbe, P., Kratzeisen, C., Lundstrom, K., Richards, J. G., Faull, R. L., Mutel, V., 1999. Cloning and functional expression of alternative spliced variants of the human metabotropic glutamate receptor 8. Molecular Brain Research 67, 201-210.

Manahan-Vaughan, D., Reymann, K. G., 1996. Metabotropic glutamate receptor subtype agonists facilitate long-term potentiation within a distinct time window in the dentate gyrus in vivo. Neuroscience 74, 723-731.

Manahan-Vaughan, D., Reymann, K. G., 1997. Group I metabotropic glutamte receptors contribute to slow-onset potentiation in the rat CA1 region *in vivo*. Neuropharmacology 36, 1533-1538.

Manev, H., Favaron, M., Guidotti, A., Costa, E., 1989. Delayed increase of Ca2+ influx elicited by glutamate: role in neuronal death. Molecular Pharmacology 36, 106-112.

Manzoni, O., Fagni, L., Pin, J.-P., Rassendren, F., Poulat, F., Sladeczek, F., Bockaert, J., 1990. (*trans*)-1-Amino-cyclopentanedicarboxylate stimulates quisqualate phosphoinositide-coupled receptors but not ionotropic glutamate receptors in striatal neurons and *Xenopus* oocytes. Molecular Pharmacology 38, 1-6.

Manzoni, O., Prezeau, L., Sladeczek, F., Bockaert, J., 1992. Trans-ACPD inhibits cAMP formation via a pertussis toxin-sensitive G-protein. European Journal of Pharmacology 225, 357-358.

Marino, M. J., Wittmann, M., Bradley, S. R., Hubert, G. W., Smith, Y., Conn, P. J., 2001. Activation of group I metabotropic glutamate receptors produces a direct excitation and disinhibition of GABAergic projection neurons in the substantia nigra pars reticulata. Journal of Neuroscience 21, 7001-7012.

Masliah, E., Alford, M., DeTeresa, R., Mallory, M., Hansen, L., 1996. Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. Annals of Neurology 40, 759-766.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., Nakanishi, S., 1991. Sequence and expression of a metabotropic glutamate receptor. Nature 349, 760-765.

Mathern, G. W., Babb, T. L., Micevych, P. E., Blanco, C. E., Pretorius, J. K., 1997. Granule cell mRNA levels for BDNF, NGF, and NT-3 correlate with neuron losses or supragranular mossy fiber sprouting in the chronically damaged and epileptic human hippocampus. Molecular and Chemical Neuropathology 30, 53-76.

Mathern, G. W., Babb, T. L., Vickrey, B. G., Melendez, M., Pretorius, J. K., 1995. The clinical-pathogenic mechanisms of hippocampal neuronal loss and surgical outcomes in temporal lobe epilepsy. Brain 118, 105-113.

Mayat, E., Lerner-Natoli, M., Rondouin, G., Lebrun, F., Sassetti, I., Recasens, M., 1994. Kainate-induced status epilepticus leads to a delayed increase in various specific glutamate receptor responses in the hippocampus. Brain Research 645, 186-200.

McBean, G. J., Roberts, P. J., 1984. Chronic infusion of L-glutamate causes neurotoxicity in rat striatum. Brain Research 290, 372-375.

McCulloch, R. M., Johnston, G. A., Game, C. J., Curtis, D. R., 1974. The differential sensitivity of spinal interneurones and Renshaw cells to Kainate and N-methyl-D-aspartate. Experimental Brain Research 21, 515-518.

McDonald, J. W., Schoepp, D. D., 1992. The metabotropic excitatory amino acid receptor agonist 1S,3R-ACPD selectively potentiates N-methyl-D-aspartate-induced brain injury. European Journal of Pharmacology 215, 353-354.

Mei, F. C., Qiao, J., Tsygankova, O. M., Meinkoth, J. L., Quilliam, L. A., Cheng, X., 2002. Differential signaling of cyclic AMP. Journal of Biological Chemistry 277, 11497-11504.

Meldrum, B. S., 1991. Excitotoxicity and epileptic brain damage. Epilepsy Research 10, 55-61.

Meldrum, B. S., Akbar, M. T., Chapman, A. G., 1999. Glutamate receptors and transporters in genetic and acquired models of epilepsy. Epilepsy Research 36, 189-204.

Meldrum, B. S., Chapman, A. G., 1999. Epileptic seizures and epilepsy. In, Basic neurochemistry: molecular, cellular and medical aspects. Eds: Siegel, G.J. et al. Lippincott-Raven Publishers, Philadelphia. pp. 755-768.

Meldrum, B. S., Garthwaite, J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends in Pharmacological Sciences 11, 379-387.

Meldrum, B. S., Craggs, M. D., Durmuller, N., S.E., S., Chapman, A. G., 1992. The effects of AMPA receptor antagonists on kindled seizures and on reflex epilepsy in rodents and primates. Epilepsy Research - Supplement 9, 307-311.

Meldrum, B. S., Ghauri, M. Tang, E., Yip, P. K., Jane, D. E., Watkins, J. C., Chapman, A.G., 1996. Glutamate metabotropic receptors: anticonvulsant and convulsant effects of group II agonists and antagonists. Society for Neuroscience Abstracts 22, 1036.

Meldrum, B. S., Vigoroux, R. A., Brierley, J. B., 1973. Systemic factors and epileptic brain damage: prolonged seizures in paralysed, artificially ventilated baboons. Archives in Neurology 29, 82-87.

Merino, M., Vizuete, M. L., Cano, J., Machado, A., 1999. The non-NMDA glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline, but not NMDA antagonists, block the intrastriatal neurotoxic effect of MPP+. Journal of Neurochemistry 73, 750-757.

Merlin, L. R., Bergold, P. J., Wong, R. K. S., 1998. Requirement of protein synthesis for group I mGluR-mediated induction of epileptiform discharges. Journal of Neurophysiology 80, 989-993.

Miller, S., Kesslak, J. P., Romano, C., Cotman, C. W., 1995. Roles of metabotropic glutamate receptors in brain plasticity and pathology. Annals of the New York Academy of Sciences 757, 460-474.

Minakami, R., Jinnai, N., Sugiyama, H., 1997. Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic in vitro. Journal of Biological Chemistry 272, 20291-20298.

Mineff, E., Valtschanoff, J., 1999. Metabotropic glutamate receptors 2 and 3 expressed by astrocytes in rat ventrobasal thalamus. Neuroscience Letters 270, 95-98.

Minchin, M. C., Beart, P. M., 1975. Compartmentation of amino acid metabolism in the rat dorsal root ganglion; a metabolic and autoradiographic study. Brain Research 83, 437-449.

Mistry, R., Golding, N., Challiss, R. A., 1998. Regulation of phosphoinositide turnover in neonatal rat cerebral cortex by group I- and II- selective metabotropic glutamate receptor agonists. British Journal of Pharmacology 123, 581-589.

Mitosumoto, H., Chad, D. A., Pioro, E. P., 1998. Excitatory and oxidative damage in ALS pathogenesis. In, Amyotropic lateral sclerosis. F. A. Davis Company, Philadelphia. pp. 197-225.

Miyamoto, M., Ishida, M., Shinozaki, H., 1997. Anticonvulsive and neuroprotective actions of a potent agonist (DCG-IV) for group II metabotropic glutamate receptors against intraventricular kainate in the rat. Neuroscience 77, 131-140.

Moldrich, R.X., Beart, P.M., 1999. Agonists for group II mGluRs and neurotoxicity in murine neuronal cultures. Neuropharmacology 38, A31.

Moldrich, R.X., Beart, P.M., 2002. Cyclic AMP regulation *in vivo* by the group II metabotropic glutamate receptor ($mGlu_{2/3}$) agonist 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate (APDC). Proceedings of the Australian Neuroscience Society 13, 197.

Moldrich, R. X., Beart, P. M., Jane, D. E., Chapman, A. G., Meldrum, B. S., 2001. Anticonvulsant activity of 3,4-dicarboxyphenylglycines in DBA/2 mice. Neuropharmacology 40, 732-735.

Moldrich, R. X., Beart, P. M., Pascoe, C. J., Cheung, N. S., 2000. Low-affinity kainate receptor agonists induce insult-dependent apoptosis and necrosis in cultured murine cortical neurons. Journal of Neuroscience Research 59, 788-796.

Monn, J. A., Valli, M. J., Massey, S. M., Hansen, M. M., Kress, T. J., Wepsiec, J. P., Harkness, A. R., Grutsch Jr, J. L., Wright, R. A., Johnson, B. G., Andis, S. L., Kingston, A. E., Tomlinson, R., Lewis, R., Griffey, K. R., Tizzano, J. P., Schoepp, D. D., 1999. Synthesis, pharmacological characterization and molecular modeling of heterobicyclic amino acids related to LY354740: Identification of LY379268 and LY389795: Two new potent, selective and systemically active agonists for group II metabotropic glutamate receptors. Journal of Medicinal Chemistry 42, 1027-1040.

Monn, J. A., Valli, M. J., Massey, S. M., Wright, R. A., Salhoff, C. R., Johnson, B. G., Howe, T., Alt, C. A., Rhodes, G. A., Robey, R. L., Griffey, K. R., Tizzano, J. P., Kallman, M. J., Helton, D. R., Schoepp, D. D., 1997. Design, synthesis and pharmacological characterization of (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): a potent, selective and orally active group 2 metabotropic glutamate receptor agonist possessing anticonvulsant and anxiolytic properties. Journal of Medicinal Chemistry 40, 528-537.

Montoliu, C., Llansola, M., Cucarella, C., Grisola, S., Felipo, V., 1997. Activation of metabotropic glutamate receptor mGluR5 prevents glutamate toxicity in primary cultures of cerebellar neurons. Journal of Pharmacology and Experimental Therapeutics 281, 643-647.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., Seeburg, P. H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529-540.

Mori-Okamoto, J., Ikeda, Y., 1986. Effect of calcium ions on the sensitivities of cultured cerebellar neurons to glutamate and aspartate. Brain Research 391, 1-10.

Mori-Okamoto, J., Okamoto, K., Sekiguchi, M., 1987. Electrophysiological and pharmacological actions of N-acetylaspartylglutamate intracellularly studied in cultured chick cerebellar neurons. Brain Research 401, 60-67.

Morita, T., Shimada, A., Ohama, E., Umemura, T., Fukuda, S., 1999. Oligodendroglial vacuolar degeneration in the bilateral motor cortices and astrocytosis in epileptic beagle dogs. Journal of Veterinary Medical Science 61, 107-111.

Mortensen, M., Suzdak, P. D., Thomsen, C., 1995. The effect of lorazepam tolerance and withdrawal on metabotropic glutamate receptor function. Journal of Pharmacology and Experimental Therapeutics 274, 155-163.

Morton, A. J., Leavens, W., 2000. Mice transgenic for the human Huntington's disease mutation have reduced sensitivity to kainic acid toxicity Brain Research Bulletin 52, 51-59.

Mouritzen-Dam, A., 1980. Epilepsy and neuron loss in the hippocampus. Epilepsia 21, 617-629.

Movsesyan, V. A., O'Leary, D. M., Fan, L., Bao, W., Mullins, P. G., Knoblach, S. M., Faden, A. I., 2001. mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine and (E)-2-methyl-6-(2-phenylethenyl)-pyridine reduce traumatic neuronal injury in vitro and in vivo by antagonizing N-methyl-D-aspartate receptors. Journal of Pharmacology and Experimental Therapeutics 296, 41-47.

Mukhin, A., Fan, L., Faden, A. I., 1996. Activation of metabotropic glutamate receptor subtype mGluR1 contributes to post-traumatic neuronal injury. Journal of Neuroscience 16, 6012-6020.

Naito, S., Ueda, T., 1985. Characterization of glutamate uptake into synaptic vesicles. Journal of Neurochemistry 44, 99-109.

Nakahara, K., Okada, M., Nakanishi, S., 1997. The metabotropic glutamate receptor mGluR5 induces calcium oscillations in cultured astrocytes via protein kinase C phosphorylation. Journal of Neurochemistry 69, 1467-1475.

Nakajima, Y., Iwakabe, H., Akazawa, C., Nawa, H., Shigemoto, R., Mizuno, N., Nakanishi, S., 1993. Molecular characterisation of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphophonobutyrate. Journal of Biological Chemistry 268, 11868-11873.

Nakanishi, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S., Okada, M., 1998. Glutamate receptors: brain function and signal transduction. Brain Research Reviews 26, 230-235.

Neki, A., Ohishi, H., Kaneko, T., Shigemoto, R., Nakanishi, S., Mizuno, N., 1996a. Pre- and postsynaptic localization of a metabotropic glutamate receptor, mGluR2, in the rat brain: an immunohistochemical study with a monoclonal antibody. Neuroscience Letters 202, 197-200.

Neki, A., Ohishi, H., Kaneko, T., Shigemoto, R., Nakanishi, S., Mizuno, N., 1996b. Metabotropic glutamate receptors mGluR2 and mGluR5 are expressed in two non-overlapping populations of golgi cells in the rat cerebellum. Neuroscience 75, 815-826.

Nicholls, D., Attwell, D., 1990. The release and uptake of excitatory amino acids. Trends in Pharmacological Sciences 11, 462-468.

Nicoletti, F., Bruno, V., Catania, M. V., Battaglia, G., Copani, A., Barbagallo, G., Cena, V., Sanchez-Prieto, J., Spano, P. F., Pizzi, M., 1999. Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. Neuropharmacology 38, 1477-1484.

Nicoletti, F., Bruno, V., Copani, A., Casabona, G., Knopfel, T., 1996. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders. Trends in Neuroscience 19, 267-271.

Nicoletti, F., Wroblewski, J. T., Novelli, A., Guidotti, A., Costa, E., 1986. Excitatory amino acid signal transduction in cerebellar cell cultures. Functional Neurology 1, 345-349.

Nicotera, P., Ankarcrona, M., Bonfoco, E., Orrenius, S., Lipton, S. A., 1997. Neuronal necrosis and apoptosis: two distinct events induced by exposure to glutamate or oxidative stress. Advances in Neurology 72, 95-101.

O'Connor, V., El Far, O., Bofill-Cardona, E., Nanoff, C., Freissmuth, M., Karschin, A., Airas, J. M., Betz, H., Boehm, S., 1999. Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. Science 286, 1180-1184.

Ogata, T., Nakamura, Y., Schubert, P., 1996. Potentiated cAMP rise in metabotropically stimulated rat cultured astrocytes by a Ca^{2+}-related A_1/A_2 adenosine receptor cooperation. European Journal of Neuroscience 8, 1124-1131.

Ogata, T., Nakamura, Y., Tsuji, K., Shibata, T., Kataoka, K., Schubert, P., 1994. Adenosine enhances intracellular Ca^{2+} mobilization in conjunction with metabotropic glutamate receptor activation by *t*-ACPD in cultured hippocampal astrocytes. Neuroscience Letters 170, 4-8.

O'Hara, P. J., Sheppard, P. O., Thorgersen, H., Venezia, D., Haldeman, B. A., McGrane, V., Houamed, K. M., Thomsen, C., 1993. The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. Neuron 11, 41-52.

Ohishi, H., Ogawa-Meguro, R., Shigemoto, R., Kaneko, T., Nakanishi, S., Mizuno, N., 1994. Immunohistochemical localisation of metabotropic glutamate receptors, mGluR2 and mGluR3, in rat cerebellar cortex. Neuron 13, 55-66.

Ohishi, H., Shigemoto, R., Nakanishi, S., Mizuno, N., 1993a. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR3) in the rat brain: an in situ hybridization study. The Journal of Comparative Neurology 335, 252-266.

Ohishi, H., Sigemoto, R., Nakanishi, S., Mizuno, N., 1993b. Distribution of the messenger RNA for a metabotropic glutamate receptor, mGluR2, in the central nervous system of the rat. Neuroscience 53, 1009-1018.

O'Leary, D. M., Movsesyan, V., Vicini, S., Faden, A. I., 2000. Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism. British Journal of Pharmacology 131, 1429-1437.

Olney, J. W., 1971. Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study. Journal of Neuropathology and Experimental Neurology 30, 75-90.

Olney, J. W., 1983. Excitotoxins: an overview. In, Excitotoxins. Eds: Fuxe, K., Roberts, P., Scwartz, R. Macmillan Press, London. pp. 82-96.

Olney, J. W., Collins, R. C., Sloviter, R. S., 1986. Excitotoxic mechanisms of epileptic brain damage. Advances in Neurology 44, 857-877.

Olney, J. W., Fuller, T., Gubareff, T., 1979. Acute dendrotoxic changes in the hippocampus of kainate treated rats. Brain Research 176, 91-100.

Olney, J. W., Labruyere, J., Price, M. T., 1990. Pathological changes induced in cerebrocortical neurons by phenycyclidine and related drugs. Science 244, 1360-1362.

Olney, J. W., Sharpe, L. G., 1969. Brain lesions in an infant rhesus monkey treated with monsodium glutamate. Science 166, 386-388.

Ongini, E., Dionisotti, S., Gessi, S., Irenius, E., Fredholm, B.B., 1999. Comparison of CGS 15943, ZM 241385 and SCH 58261 as antagonists at human adenosine receptors. Naunyn-Schmiedeberg's Archives of Pharmacology 359, 7-10.

Oppenheim, R. W., 1991. Cell death during development of the nervous system. Annual Review of Neuroscience 14, 453-501.

Ornstein, P. L., Bleisch, T. J., Arnold, M. B., Kennedy, J. H., Wright, R. A., Johnson, B. G., Tizzano, J. P., Helton, D. R., Kallman, M. J., Schoepp, D. D., 1998. 2-Substituted (2*SR*)-2-amino-2-((1*SR*,2*SR*)-2-carboxycycloprop-1-yl)glycines as potent and selective antagonists of group II metabotropic glutamate receptors. 2. Effects of aromatic substitution, pharmacological characterization, and bioavailability. Journal of Medicinal Chemistry 41, 358-378.

Pagano, A., Ruegg, D., Litschig, S., Stoehr, N., Stierlin, C., Heinrich, M., Floersheim, P., Prezeau, L., Carroll, F., Pin, J.-P., 2000. The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group-I metabotropic glutamate receptors. Journal of Biological Chemistry 275, 33750-33758.

Palmer, E., Monaghan, D. T., Cotman, C. W., 1989. Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. European Journal of Pharmacology 166, 585-587.

Palmer, M. J., Irving, A. J., Seabrook, G. R., Jane, D. E., Collingridge, G. L., 1997. The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. Neuropharmacology 36, 1517-1532.

Parmentier, M.-L., Joly, C., Restituito, S., Bockeart, J., Grau, Y., Pin, J.-P., 1998. The G protein-coupling profile of metabotropic glutamate receptors, as determined with exogenous G proteins, is independent of their ligand recognition domain. Molecular Pharmacology 53, 778-786.

Passani, L. A., Vonsattel, J. P., Coyle, J. T., 1997. Distribution of *N*-acetylaspartylglutamate immunoreactivity in human brain and its alteration in neurodegenerative disease. Brain Research 722, 9-22.

Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates. 2nd Edition. Academic Press, Sydney.

Pellicciari, R., Costantino, G., 1999. Metabotropic G-protein-coupled glutamate receptors as therapeutic targets. Current Opinion in Chemical Biology 3, 433-440.

Pellegrini-Giamprieto, D.E., Cherici, G., Alesiani, M., Carla, V., Moroni, F. 1988. Excitatory amino acid release from rat hippocampal slices as a consequence of free-radical formation. Journal of Neurochemistry 51, 1960-1963.

Pellegrini-Giampietro, D. E., Cozzi, A., Peruginelli, F., Leonardi, P., Meli, E., Pellicciari, R., Moroni, F., 1999. 1-Aminoindan-1,5-dicarboxylic acid and (S)-(+)-2-(3'-carboxybicyclo[1.1.1] pentyl)-glycine, two mGlu1 receptor-preferring antagonists, reduce neuronal death in in vitro and in vivo models of cerebral ischaemia. European Journal of Neuroscience 11, 3637-3647.

Perroy, J., El Far, O., Bertaso, F., Pin, J.-P., Betz, H., Bockaert, J., Fagni, L., 2002. PICK1 is required for the control of synaptic transmission by the metabotropic glutamate receptor 7. EMBO Journal 21, 2990-2999.

Phillips, T., Makoff, A., Brown, S., Rees, S., Emson, P., 1997. Localization of mGluR4 protein in the rat cerebral cortex and hippocampus. NeuroReport 8, 3349-3354.

Philpott, K. L., McCarthy, M. J., Becker, D., Gatchalian, C., Rubin, L. L., 1996. Morphological and biochemical changes in neurons: apoptosis versus mitosis. European Journal of Neuroscience 8, 1906-1915.

Pin, J.-P., De Colle, C., Bessis, A. S., Acher, F., 1999. New perspectives for the development of selective metabotropic glutamate receptor ligands. European Journal of Pharmacology 375, 277-294.

Pin, J.-P., Duvoisin, R., 1995. The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34, 1-26.

Pisani, A., Calabresi, P., Centonze, D., Bernardi, G., 1997. Enhancement of NMDA responses by group I metabotropic glutamate receptor activation in striatal neurones. British Journal of Pharmacology 120, 1007-1014.

Pizzi, M., Benarese, M., Boroni, F., Goffi, F., Valerio, A., Spano, P. F., 2000. Neuroprotection by metabotropic glutamate receptor agonists on kainate-induced degeneration of motor neurons in spinal cord slices from adult rat. Neuropharmacology 39, 903-910.

Pizzi, M., Boroni, F., Moraitis, K., Bianchetti, A., Memo, M., Spano, P., 1999. Reversal of glutamate excitotoxicity by activation of PKC-associated metabotropic glutamate receptors in cerebellar granule cells relies on NR2C subunit expression. European Journal of Neuroscience 11, 2489-2496.

Pizzi, M., Consolandi, O., Memo, M., Spano, P. F., 1996. Activation of multiple metabotropic glutamate receptor subtypes prevents NMDA-induced excitotoxicity in rat hippocampal slices. European Journal of Neuroscience 8, 1516-1521.

Pizzi, M., Fallacara, C., Arrighi, V., Memo, M., Spano, P., 1993. Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary cultures of cerebellar granule cells. Journal of Neurochemistry 61, 683-689.

Pizzi, M., Ribola, M., Valerio, A., Memo, M., Spano, P., 1991. Various Ca2+ entry blockers prevent glutamate-induced neurotoxicity. European Journal of Pharmacology 209, 169-173.

Pollard, H., Charriaut-Marlangue, C., Cantagrel, S., Represa, A., Robain, O., Moreau, J., Ben-Ari, Y., 1994. Kainate-induced apoptotic cell death in hippocampal neurons. Neuroscience 63, 369-377.

Pook, P. C.-K., Birse, E. F., Jane, D. E., Jones, A. W., Jones, P. L. S.-J., Mewett, K. N., Sunter, D. C., Udvarhelyi, P. M., Wharton, B., Watkins, J. C., 1993. Differential actions of the metabotropic glutamate receptor antagonists 4C-PG and M4C-PG at L-AP4-like receptors in neonatal rat spinal cord. British Journal of Pharmacology Proceedings Supplement 108, 87P.

Popoli, P., Pezzola, A., Torvinen, M., Reggio, R., Pintor, A., Scarchilli, L., Fuxe, K., Ferre, S., 2001. The selective mGlu(5) receptor agonist CHPG inhibits quinpirole-induced turning in 6-hydroxydopamine-lesioned rats and modulates the binding characteristics of dopamine D(2) receptors in the rat striatum: interactions with adenosine A(2a) receptors. Neuropsychopharmacology 25, 505-513.

Portera-Cailliau, C., Price, D. L., Martin, L. J., 1997. Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. Journal of Comparative Neurology 378, 70-87.

Pow, D. V., Barnett, N. L., 2000. Developmental expression of excitatory amino acid transporter 5: a photoreceptor and bipolar cell glutamate transporter in rat retina. Neuroscience Letters 280, 21-24.

Prezeau, L., Carrette, J., Helpap, B., Curry, K., Pin, J.-P., Bockeart, J., 1994. Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures. Molecular Pharmacology 45, 570-577.

Prezeau, L., Gomeza, J., Ahern, S., Mary, S., Galvez, T., Bockeart, J., Pin, J.-P., 1996. Changes in the carboxyl-terminal domain of metabotropic glutamate receptor 1 by alternative splicing generate receptors with differing agonist-independent activity. Molecular Pharmacology 49, 422-429.

Prezeau, L., Manzoni, O., Homburger, V., Sladeczeck, F., Curry, K., Bockaert, J., 1992. Characterization of a metabotropic glutamate receptor: direct negative coupling to adenylate cyclase and involvement of a pertussis toxin-sensitive G-protein. Proceedings of the National Academy of Sciences of the United States of America 89, 8040-8044.

Qin, Z. H., Wang, Y., Chase, T. N., 1996. Stimulation of N-methyl-D-aspartate receptors induces apoptosis in rat brain. Brain Research 725, 166-176.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation: II Motor seizure. Electroencephalography and Clinical Neurophysiology 32, 281-294.

Rahman, S., Neuman, R. S., 1996. Characterization of metabotropic glutamate receptor-mediated facilitation of N-methyl-D-aspartate depolarization of neocortical neurones. British Journal of Pharmacology 117, 675-683.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacological Reviews 50, 413-492.

Recasens, M., Sassetti, I., Nourigat, A., Sladeczek, F., Bockaert, J., 1987. Characterization of subtypes of excitatory amino acid receptors involved in the stimulation of inositol phosphate synthesis in rat brain synaptoneurosomes. European Journal of Pharmacology 141, 87-93.

Renger, J. J., Hartman, K. N., Tsuchimoto, Y., Yokoi, M., Nakanishi, S., Hensch, T. K., 2002. Experience-dependent plasticity without long-term depression by type 2 metabotropic glutamate receptors in developing visual cortex. Proceedings of the National Academy of Sciences of the United States of America 99, 1041-1046.

Ribeiro, J.A., 1999. Adenosine A_{2A} receptor interactions with receptors for other neurotransmitters and neuromodulators. European Journal of Pharmacology 375, 101-113.

Richardson-Burns, S. M., Haroutunian, V., Davis, K. L., Watson, S. J., Meador-Woodruff, J. H., 2000. Metabotropic glutamate receptor mRNA expression in the schizophrenic thalamus. Biological Psychiatry 47, 22-28.

Rodriguez-Moreno, A., Sistiaga, A., Lerma, J., Sanchez-Prieto, J., 1998. Switch from facilitation to inhibition of excitatory synaptic transmission by group I mGluR desensitization. Neuron 21, 1477-1486.

Rogawski, M. A., 1993. Therapeutic potential of excitatory amino acid antagonists: channel blockers and 2,3-benzodiazepines. Trends in Pharmacological Sciences 14, 325-331.

Romano, C., Van Den Pol, A. N., O'Malley, K. L., 1996. Enhanced early developmental expression of the metabotropic glutamate receptor mGlu5 in rat brain: protein, mRNA splice variants, and region distribution. Journal of Comparative Neurology 367, 403-412.

Rosdahl, D., Seitzberg, D. A., Christensen, T., Balchen, T., Diemer, N. H., 1994. Changes in mRNA for metabotropic glutamate receptors after transient cerebral ischaemia. NeuroReport 5, 593-596.

Roth, K. A., D'Sa, C., 2001. Apoptosis and brain development. Mental Retardation and Developmental Disabilities Research Reviews 7, 261-266.

Ro[illegible], F. M., Casssidy, J., Wilson, M., Davies, S. N., 2000. Developmental regulation of hippocampal excitatory synaptic transmission by metabotropic glutamate receptors. British Journal of Pharmacology 131, 453-464.

Sabelhaus, C. F., Schroder, U. H., Breder, J., Henrich-Noack, P., Reymann, K. G., 2000. Neuroprotection against hypoxic/hypoglycaemic injury after the insult by the group III metabotropic glutamate receptor agonist (R,S)-4-phosphonophenylglycine. British Journal of Pharmacology 131, 655-658.

Sala, C., Piech, V., Wilson, N. R., Passafaro, M., Liu, G., Sheng, M., 2001. Regulation of dendritic spine morphology and synaptic function by Shank and Homer. Neuron 31, 115-130.

Salcman, M., Defendini, R., Correll, J., Gilman, S., 1978. Neuropathological changes in cerebellar biopsies of epileptic patients. Annals of Neurology 3, 10-19.

Sansig, G., Bushell, T. J., Clarke, V. R. J., Rozov, A., Burnashev, N., Portet, C., Gasparini, F., Schmutz, M., Klebs, K., Shigemoto, R., Flor, P. J., Kuhn, R., Knopfel, T., Schroeder, M., Collett, V. J., Collingridge, G. L., Van Der Putten, H., 1999. Epilepsy in mice lacking the metabotropic glutamate receptor mGlu7. Neuropharmacology 38, A39.

Saransaari, P., Oja, S. S., 1999. Involvement of metabotropic glutamate receptors in taurine release in the adult and developing mouse hippocampus. Amino Acids 16, 165-179.

Sareen, D., 2002. Neuroprotective agents in acute ischemic stroke. Journal of the Association of Physicians of India 50, 250-258.

Sastry, P. S., Rao, K. S., 2000. Apoptosis and the nervous system. Journal of Neurochemistry 74, 1-20.

Saugstad, J. A., Kinzie, J. M., Shinohara, M. M., Segerson, T. P., Westbrook, G. L., 1997. Cloning and expression of rat metabotropic glutamate receptor 8 reveals a distinct pharmacological profile. Molecular Pharmacology 51, 119-125.

Saugstad, J. A., Segerson, T. P., Westbrook, G. L., 1995. L-2-amino-3-phosphonopropionic acid competitively antagonizes metabotropic glutamate receptors 1a and 5 in *Xenopus* oocytes. European Journal of Pharmacology 289, 395-397.

Saugstad, J. A., Segerson, T. P., Westbrook, G. L., 1996. Metabotropic glutamate receptors activate G-protein-coupled inwardly rectifying potassium channels in Xenopus oocytes. Journal of Neuroscience 16, 5979-5985.

Savic, I., Seitz, R. J., Pauli, S., 1998. Brain distortions in patients with primarily generalized tonic-clonic seizures. Epilepsia 39, 364-370.

Savill, J., Fadok, V., Henson, P., Haslett, C., 1993. Phagocyte recognition of cells undergoing apoptosis. Immunology Today 14, 131-136.

Savolainen, K. M., Loikkanen, J., Naarala, J., 1995. Amplification of glutamate-induced oxidative stress. Toxicology Letters 82-83, 399-405.

Scanziani, M., Salin, P. A., Vogt, K. E., Malenka, R. C., Nicoll, R. A., 1997. Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385, 630-634.

Schaffhauser, H., de Barry, J., Muller, H., Heitz, M. P., Gombos, G., Mutel, V., 1997. Involvement of a cyclic-AMP pathway in group I metabotropic glutamate receptor responses in neonatal rat cortex. European Journal of Pharmacology 334, 289-297.

Schaffhauser, H., Cai, Z., Hubalek, F., Macek, T. A., Pohl, J., Murphy, T. J., Conn, P. J., 2000. cAMP-dependent protein kinase inhibits mGluR2 coupling to G-proteins by direct receptor phosphorylation. Journal of Neuroscience 20, 5663-5670.

Schaffhauser, H., Knoflach, F., Pink, J. R., Bleuel, Z., Cartmell, J., Goepfert, F., Kemp, J. A., Richards, J. G., Adam, G., Mutel, V., 1998. Multiple pathways for regulation of the KCl-induced [3H]-GABA release by metabotropic glutamate receptors, in primary rat cortical cultures. Brain Research 782, 91-104.

Schlag, B. D., Vondrasek, J. R., Munir, M., Kalandadze, A., Zelenaia, O. A., Rothstein, J. D., Robinson, M. B., 1998. Regulation of the glial Na^+-dependent glutamate transporters by cyclic AMP analogs and neurons. Molecular Pharmacology 53, 355-369.

Schmidt, M., Evellin, S., Oude Weernink, P.A., vom Dorp, F., Rehmann, H., Lomasney, J.W., Jakobs, K.H., 2001. A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. Nature Cell Biology 3, 1020-1024.

Schmitt, A., Asan, E., Lesch, K. P., Kugler, P., 2002. A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. Neuroscience 109, 45-61.

Schmutz, M., Arthur, A. Faleck, H., Karlsson, G., Kotake, A., Lantwicki, L., LaRue, L., Markabi, S., Murphy, D., Powell, M., Sauer, D., 1997. Selfotel (CGS19755). In, Excitatory Amino Acids. Clinical results with antagonists. Ed: Herrling, P. Academic Press, London. pp. 1-6.

Schoepp, D. D., Jane, D. E., Monn, J. A., 1999. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. Neuropharmacology 38, 1431-1476.

Schoepp, D. D., Johnson, B. G., Monn, J. A., 1992b. Inhibition of cyclic AMP formation by a selective metabotropic glutamate receptor agonist. Journal of Neurochemistry 58, 1184-1186.

Schoepp, D. D., Johnson, B. G., Monn, J. A., 1996a. (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid-induced increases in cyclic AMP formation in the neonatal rat hippocampus are mediated by a synergistic interaction between phosphoinositide- and inhibitory cyclic AMP-coupled mGluRs. Journal of Neurochemistry 66, 1981-1985.

Schoepp, D. D., Johnson, B. G., Sacaan, A. I., True, R. A., Monn, J. A., 1992a. *In vitro* and *in vivo* pharmacology of 1S,3R- and 1R,3S-ACPD: evidence for a role of metabotropic glutamate receptors in striatal motor function. Molecular Neuropharmacology 2, 33-37.

Schoepp, D. D., Johnson, B. G., Salhoff, C. R., McDonald, J. W., Johnston, M. V., 1991a. In vitro and in vivo pharmacology of trans- and cis-(±)-1-amino-1,3-cyclopentanedicaboxylic acid: dissociation of metabotropic and ionotropic excitatory amino acid receptor effects. Journal of Neurochemistry 56, 1789-1796.

Schoepp, D. D., Johnson, B. G., Salhoff, C. R., Valli, M. J., Desai, M. A., Burnett, J. P., Mayne, N. G., Monn, J. A., 1995. Selective inhibition of forskolin-stimulated cyclic AMP formation in rat hippocampus by a novel mGluR agonist, 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate. Neuropharmacology 34, 843-850.

Schoepp, D. D., Johnson, B. G., True, R. A., Monn, J. A., 1991b. Comparison of (1S,3R)-1-aminocyclopentanedicarboxylic acid (1S,3R-ACPD)- and 1R,3S-ACPD-stimulated brain phosphoinositide hydrolysis. European Journal of Pharmacology 207, 351-353.

Schoepp, D. D., Johnson, B. G., Wright, R. A., Salhoff, C. R., Mayne, N. G., Wu, S., Cockerham, S. L., Burnett, J. P., Belegaje, R., Bleakman, D., Monn, J. A., 1997. LY354740 is a potent and highly selective group II metabotropic glutamate receptor agonist in cells expressing human glutamate receptors. Neuropharmacology 36, 1-11.

Schoepp, D. D., Goldsworthy, J., Johnson, B. G., Salhoff, C. R., Baker, S. R., 1994. 3,5-Dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. Journal of Neurochemistry 63, 769-772.

Schoepp, D. D., Hillman, C. C., 1990. Developmental and pharmacological characterization of quisqualate, ibotenate, and trans-1-amino-1,3-cyclopentanedicarboxylic acid stimulations of phosphoinositide hydrolysis in rat cortical brain slices. Biogenic Amines 7, 331-340.

Schoepp, D. D., Salhoff, C. R., Wright, R. A., Johnson, B. G., Burnett, J. P., Mayne, N. G., Belagaje, R., Wu, S., Monn, J. A., 1996b. The novel metabotropic glutamate receptor agonist 2*R*,4*R*-(2R,4R)-APDC potentiates stimulation of phosphoinositide hydrolysis in the rat hippocampus by 3,5-dihydroxyphenyglycine: evidence for a synergistic interaction between group 1 and group 2 receptors. Neuropharmacology 35, 1661-1672.

Schousboe, A., 1981. Transport and metabolism of glutamate and GABA in neuron and glial cells. International Review of Neurobiology 22, 1-45.

Schubert, P., Ogata, T., Ferroni, S., McRae, A., Nakamura, Y., Rudolphi, K., 1996. Modulation of glial cell signaling by adenosine and pharmacological reinforcement. A neuroprotective strategy? Molecular and Chemical Neuropathology 28,185-90.

Schubert, P., Ogata, T., Marchini, C., Ferroni, S., 2001. Glia-related pathomechanisms in Alzheimer's disease: a therapeutic target? Mechanisms of Ageing and Development 123, 47-57.

Schubert, P., Ogata, T., Marchini, C., Ferroni, S., Rudolphi, K., 1997. Protective mechanisms of adenosine in neurons and glial cells. Annals of the New York Academy of Sciences 825, 1-10.

Scott, H. L., Tannenberg, A. E., Dodd, P. R., 1995. Variant forms of neuronal glutamate transporter sites in Alzheimer's disease cerebral cortex. Journal of Neurochemistry 64, 2193-2202.

Sekiguchi, M., Okamoto, K., Sakai, Y., 1987. Excitatory action of N-acetylaspartylglutamate on Purkinje cells in guinea pig cerebellar slices: an intrasomatic study. Brain Research 423, 23-33.

Selbie, L. A., Hill, S. J., 1998. G protein-coupled-receptor cross talk: the fine-tuning of multiple receptor-signalling pathways. Trends in Pharmacological Sciences 19, 87-93.

Sharon, D., Vorobiov, D., Dascal, N., 1997. Positive and negative coupling of the metabotropic glutamate receptors to a G protein-activated K+ channel, GIRK, in Xenopus oocytes. Journal of General Physiology 109, 477-490.

Shastry, B. S., 2001. Molecular and cell biological aspects of Alzheimer disease. Journal of Human Genetics 46, 609-618.

Shave, E., Pliss, L., Lawrance, M. L., FitzGibbon, T., Stastny, F., Balcar, V. J., 2000. Regional distribution and pharmacological characteristics of [^{3}H]N-acetyl-aspartyl-glutamate (NAAG) binding sites in rat brain. Neurochemistry International 38, 53-62.

Sheardown, M. J., 1988. A new and specific non-NMDA receptor antagonist, FG 9065, blocks L-AP4-evoked depolarization in rat cerebral cortex. European Journal of Pharmacology 148, 471-474.

Shigemoto, R., Mizuno, N., 2000. Metabotropic glutamate receptors - immunocytochemical and in situ hybridization analyses. In, Glutamate. Handbook of Chemical Neuroanatomy. Eds: Ottersen, O.P., Storm-Mathisen, J. Elsevier, Amsterdam. pp. 63-98.

Shigemoto, R., Nakanishi, S., Mizuno, N., 1992. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. Journal of Comparative Neurology 322, 121-135.

Shupliakov, O., Brodin, L., Gullheim, S., Ottersen, O. P., Storm-Mathisen, J., 1992. Immunogold quantification of glutamate in two types of excitatory synapse with different firing patterns. Journal of Neuroscience 12, 3789-3803.

Siesjo, B. K., 1990. Calcium in the brain under physiological and pathological conditions. European Neurology 30, 3-9.

Siesjo, B. K., Bengtsson, F., Grampp, W., Theander, S., 1989. Calcium, excitotoxins, and neuronal death in the brain. Annals of the New York Academy of Sciences 568, 234-251.

Siliprandi, R., Lipartiti, M., Fadda, E., Sautter, J., Manev, H., 1992. Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. European Journal of Pharmacology 219, 173-174.

Simonian, N. A., Getz, R. L., Leveque, J. C., Konradi, C., Coyle, J. T., 1996. Kainic acid induces apoptosis in neurons. Neuroscience 75, 1047-1055.

Sims, K. D., Robinson, M. B., 1999. Expression patterns and regulation of glutamate transporters in the developing and adult nervous system. Critical Reviews in Neurobiology 13, 169-197.

Sladeczek, F., Pin, J.-P., Recasens, M., Bockaert, J., Weiss, S., 1985. Glutamate stimulates inositol phosphate formation in striatal neurones. Nature 317, 717-719.

Small, D. L., Buchan, A. M., 1997. NMDA antagonists: their role in neuroprotection In Neuroprotective agents and cerebral ischaemia. Eds: Green, A. R., Cross, A. J. Academic Press, London. pp. 137-172.

Small, D. L., Monette, R., Chakravarthy, B., Durkin, J., Barbe, G., Mealing, G., Morley, P., Buchan, A. M., 1996. Mechanisms of 1*S*,3*R*-ACPD-induced neuroprotection in rat hippocampal slices subjected to oxygen and glucose deprivation. Neuropharmacology 35, 1037-1048.

Sorensen, S. D., Macek, T. A., Cai, Z., Saugstad, J. A., Conn, P. J., 2002. Dissociation of protein kinase-mediated regulation of metabotropic glutamate receptor 7 (mGluR7) interactions with calmodulin and regulation of mGluR7 function. Molecular Pharmacology 61, 1303-1312.

Speliotes, E. K., Hartnett, K. A., Blitzblau, R. C., Aizenman, E., Rosenberg, P. A., 1994. Comparison of the potency of competitive NMDA antagonists against the neurotoxicity of glutamate and NMDA. Journal of Neurochemistry 63, 879-885.

Spooren, W. P., Gasparini, F., Bergmann, R., Kuhn, R., 2000. Effects of the prototypical mGlu(5) receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine on rotarod, locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats. European Journal of Pharmacology 406, 403-410.

Steller, H., 1995. Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

Stephan, D., Bon, C., Holzwarth, J. A., Galvan, M., Pruss, R. M., 1996. Human metabotropic glutamate receptor 1: mRNA distribution, chromosome localization and functional expression of two splice variants. Neuropharmacology 35, 1649-1660.

Stoessl, A. J., 1999. Etiology of Parkinson's disease. Canadian Journal of Neurological Sciences 26, S5-12.

Storm-Mathisen, J., Ottersen, O. P., 1987. Tracing of neurons with glutamate or gamma-aminobutyrate as putative transmitters. Biochemical Society Transactions 15, 210-213.

Storm-Mathisen, J., Danbolt, N. C., Ottersen, O. P., 1995. Localization of glutamate and its membrane transport proteins. In, CNS neurotransmitters and neuromodulators: Glutamate. Eds: Stone, T. W. CRC Press, New York. pp. 1-18.

Sugita, S., Sudhof, T.C., 2000. Specificity of Ca2+-dependent protein interactions mediated by the C2A domains of synaptotagmins. Biochemistry 39, 2940-2949.

Sun, J., Tadokoro, S., Imanaka, T., Murakami, S. D., Nakamura, M., Kashiwada, K., Ko, J., Nishida, W., Sobue, K., 1998. Isolation of PSD-Zip45, a novel Homer/vesl family protein containing leucine zipper motifs, from rat brain. FEBS Letters 437, 304-308.

Sutula, T., 2002. Antiepileptic drugs prevent neural degeneration associated with epilepsy: assessing the prospects for neuroprotection. Epilepsy Research 50, 125-129.

Suzuki, T., Lu, M.-S., Motegi, H., Yoshii, T., Misawa, M., 1992. Genetic differences in the development of physical dependence upon diazepam in Lewis and Fischer 344 inbred rat strains. Pharmacology and Biochemisty of Behaviour 43, 387-393.

Suzuki, K., Mori, N., Kittaka, H., Iwata, Y., Yamada, Y., Osonoe, K., Niwa, S., 1996. Anticonvulsant action of metabotropic glutamate receptor agonists in kindled amygdala of rats. Neuroscience Letters 204, 41-44.

Suzuki, T., Shimizu, N., Tsuda, M., Soma, M., Misawa, M., 1999. Role of metabotropic glutamate receptors in the hypersusceptibility to pentylenetetrazole-induced siezure during diazepam withdrawal. European Journal of Pharmacology 369, 163-168.

Taber, M. T., Das, S., Fibiger, H. C., 1995. Cortical regulation of subcortical dopamine release: mediation via the ventral tegmental area. Journal of Neurochemistry 65, 1407-1410.

Takahashi, K., Tsuchida, K., Tanabe, Y., Masu, M., Nakanishi, S., 1993. Role of the large extracellular domain of metabotropic glutamate receptors in agonist selectivity determination. Journal of Biological Chemistry 268, 19341-19345.

Takeda, S., Wakabayashi, K., Ohama, E., Ikuta, F., 1988. Neuropathology of myoclonus epilepsy associated with ragged-red fibers (Fukuhara's disease). Acta Neuropathologica 75, 433-440.

Tamaru, Y., Nomura, S., Mizuno, N., Shigemoto, R., 2001. Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: differential location relative to pre- and postsynaptic sites. Neuroscience 106, 481-503.

Tanabe, Y., Nomura, A., Masu, M., Shigemoto, R., Mizuno, N., Nakanishi, S., 1993. Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. Journal of Neuroscience 13, 1372-1378.

Tanaka, O., Sakagami, H., Kondo, H., 1995. Localization of mRNAs of voltage-dependent Ca(2+)-channels: four subtypes of alpha 1- and beta-subunits in developing and mature rat brain. Molecular Brain Research 30, 1-16.

Tang, E., Yip, P. K., Chapman, A. G., Jane, D. E., Meldrum, B. S., 1997. Prolonged anticonvulsant action of glutamate metabotropic receptor agonists in inferior colliculus of genetically epilepsy-prone rats. European Journal of Pharmacology 327, 109-115.

Tang, F. R., Lee, W. L., 2001. Expression of the group II and III metabotropic glutamate receptors in the hippocampus of patients with mesial temporal lobe epilepsy. Journal of Neurocytology 30, 137-143.

Tang, F. R., Lee, W. L., Yang, J., Sim, M. K., Ling, E. A., 2001b. Expression of metabotropic glutamate receptor 8 in the rat hippocampus after pilocarpine induced status epilepticus. Neuroscience Letters 300, 137-140.

Tang, F. R., Lee, W. L., Yang, J., Sim, M. K., Ling, E. A., 2001c. Expression of metabotropic glutamate receptor 1 in the hippocampus of rat pilocarpine induced status epilepticus. Epilepsy Research 46, 179-189.

Tang, F. R., Lee, W. L., Yeo, T. T., 2001a. Expression of the group I metabotropic glutamate receptor in the hippocampus of patients with mesial temporal lobe epilepsy. Journal of Neurocytology 30, 403-411.

Taylor, S. S., Buechler, J. A., Yonemoto, W., 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annual Review of Biochemistry 59, 971-1005.

Teitelbaum, J. S., Zatore, R. J., Carpenter, S., Gendron, D., Evans, A. C., Gjedde, A., Cashman, N. R., 1990. Neurologic sequele of domoic acid intoxication due to the ingestion of contaminated mussels. New England Journal of Medicine 322, 1781-1787.

Tesmer, J. J. G., Sprang, S. R., 1998. The structure, catalytic mechanism and regulation of adenylyl cyclase. Current Opinion in Structural Biology 8, 713-719.

Thomas, N. K., Wright, R. A., Howson, P. A., Kingston, A. E., Schoepp, D. D., Jane, D. E., 2001. (S)-3,4-DCPG, a potent and selective mGlu8a receptor agonist, activates metabotropic glutamate receptors on primary afferent terminals in the neonatal rat spinal cord. Neuropharmacology 40, 311-318.

Thomsen, C., Bruno, V., Nicoletti, F., Marinozzi, M., Pellicciari, R., 1996. (2*S*,1'*S*,2'*S*,3'*R*)-2-(2'-Carboxy-3'-phenylcyclopropyl)glycine, a potent and selective antagonist of type 2 metabotropic glutamate receptors. Molecular Pharmacology 50, 6-9.

Thomsen, C., Frandsen, A., Suzdak, P. D., Andersen, C. F., Schousboe, A., 1993. Effects of t-ACPD on neural survival and second messengers in cultured cerebral cortical neurones. NeuroReport. 4, 1255-1258.

Thomsen, C., Klitgaard, H., Sheardown, M., Jackson, H. C., Eskesen, K., Jacobsen, P., Treppendahl, S., Suzak, P. D., 1994. (*S*)-4-Carboxy-3-hydoxyphenylglycine, an antagonist of metabotropic glutamate receptor (mGluR)1a and an agonist of mGluR2, protects against audigenic seizures in DBA/2 mice. Journal of Neurochemistry 62, 2492-2495.

Tizzano, J. P., Griffey, K. I., Schoepp, D. D., 1995. Induction or protection of limbic seizures in mice by mGluR subtype selective agonists. Neuropharmacology 34, 1063-1067.

Tones, M. A., Bendali, N., Flor, P. J., Knophel, T., Kuhn, R., 1995. The agonist selectivity of a class III metabotropic glutamate receptor, human mGluR4a, is determined by the N-terminal extracellular domain. NeuroReport 7, 117-120.

Treiman, M., Caspersen, C., Christensen, S. B., 1998. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases. Trends in Pharmacological Sciences 19, 131-135.

Tse, F. W., Fraser, D. D., Duffy, S., MacVicar, B. A., 1992. Voltage-activated K+ currents in acutely isolated hippocampal astrocytes. Journal of Neuroscience 12, 1781-1788.

Tu, J. C., Xiao, B., Yuan, J. P., Lanahan, A. A., Leoffert, K., Li, M., Linden, D. J., Worley, P. F., 1998. Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron 21, 717-726.

Ugolini, A., Corsi, M., Bordi, F., 1997. Potentiation of NMDA and AMPA responses by group I mGluR in spinal cord motoneurons. Neuropharmacology 36, 1047-1055.

Ulas, J., Satou, T., Ivins, K. J., Kesslak, J. P., Cotman, C. W., Balazs, R., 2000. Expression of metabotropic glutamate receptor 5 is increased in astrocytes after kainate-induced epileptic seizures. Glia 30, 352-361.

van den Pol, A. N., Kogelman, L., Ghosh, P., Liljelund, P., Blackstone, C., 1994. Developmental regulation of the hypothalamic metabotropic glutamate receptor mGluR1. Journal of Neuroscience 14, 3816-3834.

van den Pol, A. N., Obrietan, K., Belousov, A., 1996. Glutamate hyperexcitability and seizure-like activity throughout the brain and spinal cord upon relief from chronic glutamate receptor blockade in culture. Neuroscience 74, 653-674.

van den Pol, A. N., Romano, C., Ghosh, P., 1995. Metabotropic glutamate receptor mGluR5 subcellular distribution and developmental expression in hypothalamus. Journal of Comparative Neurology 362, 134-150.

Vandenberg, R. J., 1998. Molecular pharmacology and physiology of gluatamate transporters in the central nervous system. Clinical and Experimental Pharmacology and Physiology 25, 393-400.

Vaux, D. L., Cory, S., Adams, J. M., 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335, 440-442.

Vazquez, E., Budd, D. C., Herrero, I., Nicholls, D. G., Sanchez-Prieto, J., 1995. Co-existence and interaction between facilitatory and inhibitory metabotropic glutamate receptors and the inhibitory adenosine A_1 receptor in cerebrocortical nerve terminals. Neuropharmacology 34, 919-927.

Vazquez, E., Herrero, I., Miras-Portugal, M. T., Sanchez-Prieto, J., 1994. Role of arachidonic acid in the facilitation of glutamate release from rat cerebrocortical synaptosomes independent of metabotropic glutamate receptor responses. Neuroscience Letters 174, 9-13.

Verkhratsky, A., Steinhäuser, C., 2000. Ion channels in glial cells. Brain Research Reviews 32, 380-412.

Vicini, S., Rumbaugh, G., 2000. A slow NMDA channel: in search of a role. Journal of Physiology 525, 283.

Walaas, I., 1981. Biochemical evidence for overlapping neocortical and allocortical glutamate projections to the nucleus accumbens and rostral caudatoputamen in the rat brain. Neuroscience 6, 399-405.

Walker, J. E., Fonnum, F., 1983. Regional cortical glutamergic and aspartergic projections to the amygdala and thalamus of the rat. Brain Research 261, 371-374.

Wang, J., Lonart, G., Johnson, K. M., 1996. Glutamate receptor activation induces carrier mediated release of endogenous GABA from rat striatal slices. Journal of Neural Transmission - General Section 103, 31-43.

Watkins, J., Collingridge, G., 1994. Phenylglycine derivatives as antagonists of metabotropic glutamate receptors. Trends in Pharmacological Sciences 15, 333-342.

Waxman, S. G., 1996. Correlative Neuroanatomy (Edition 23). Appleton and Lange, Oxford. pp. 413.

Wei, J., Wayman, G., Storm, D. R., 1996. Phosphorylation and inhibition of type III adenylyl cyclase by calmodulin-dependent protein kinase II in vivo. Journal of Biological Chemistry 271, 24231-24235.

Weiss, J. H., Hartley, D. M., Koh, J., Choi, D. W., 1990. The calcium channel blocker nifedipine attenuates slow excitatory amino acid neurotoxicity. Science 247, 1474-1477.

Westbrook, G. L., Mayer, M. L., Namboodiri, M. A., Neale, J. H., 1986. High concentrations of N-acetylaspartylglutamate (NAAG) selectively activate NMDA receptors on mouse spinal cord neurons in cell culture. Journal of Neuroscience 6, 3385-3392.

Whittemore, E. R., Koerner, J. F., 1989a. Novel recognition site for L-quisqualate sensitizes neurons to depolarization by L-2-amino-4-phosphonobutanoate (L-AP4). Brain Research 489, 146-156.

Whittemore, E. R., Koerner, J. F., 1989b. An explanation for the purported excitation of piriform cortical neurons by N-acetyl-L-aspartyl-L-glutamic acid (NAAG). Proceedings of the National Academy of Sciences of the United States of America 86, 9602-9605.

Whittemore, E. R., Loo, D. T., Cotman, C. W., 1994. Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. Neuroreport 5, 1485-1488.

Winder, D. G., Conn, J. P., 1992. Activation of metabotropic glutamate receptors in the hippocampus increases cyclic AMP accumulation. Journal of Neurochemistry 59, 375-378.

Winder, D. G., Conn, J. P., 1995. Metabotropic glutamate receptor (mGluR)-mediated potentiation of cyclic AMP responses does not require phosphoinositdie hydrolysis: mediation by a group II-like mGluR. Journal of Neurochemistry 64, 592-599.

Winder, D. G., Conn, J. P., 1996. Roles of metabotropic glutamate receptors in glial function and glial-neuronal communication. Journal of Neuroscience Research 46, 131-137.

Wisden, W., Seeburg, P. H., Monyer, H., 2000. AMPA, kainate and NMDA ionotropic glutamate receptor expression - an in situ hybridization atlas. In, Glutamate: Handbook of Chemical Neuroanatomy. Eds: Ottersen, O.P., Storm-Mathisen, J. Elsevier, Amsterdam. pp. 99-144.

Wroblewska, B., Wroblewski, J. T., Pshenichkin, S., Surin, A., Sullivan, S. E., Neale, J. H., 1997. N-acetylaspartylglutamate selectively activates mGluR3 receptors in transfected cells. Journal of Neurochemistry 69, 174-181.

Wu, M. L., Chen, W. H., Lui, I. H., Tseng, C. D., Wang, S. M., 1999. A novel effect of cAMP on capacitative Ca^{2+} entry in cultured rat cerebellar astrocytes. Journal of Neurochemistry 73, 1318-1328.

Wu, S., Wright, R. A., Rockey, P. K., Burgett, S. G., Arnold, J. S., Rosteck, P. R., Jr., Johnson, B. G., Schoepp, D. D., Belagaje, R. M., 1998. Group III human metabotropic glutamate receptors 4, 7 and 8: molecular cloning, functional expression, and comparison of pharmacological properties in RGT cells. Molecular Brain Research 53, 88-97.

Wyllie, A. H., Kerr, J. F., Currie, A. R., 1980. Cell death: the significance of apoptosis. International Review of Cytology 68, 251-306.

Wyllie, A. H., Morris, R. G., Smith, A. L., Dunlop, D., 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. Journal of Pathology 142, 67-77.

Xiao, B., Tu, J. C., Petralia, R. S., Yuan, J. P., Doan, A., Breder, C. D., Ruggiero, A., Lanahan, A. A., Wenthold, R. J., Worley, P. F., 1998. Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. Neuron 21, 707-716.

Yokoi, M., Kobayashi, K., Manabe, T., Takahashi, T., Sakaguchi, I., Katsuura, G., Shigemoto, R., Ohishi, H., Nomura, S., Nakamura, K., Nakao, K., Katsuki, M., Nakanishi, S., 1996. Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. Science 273, 645-647.

Young, A. B., Greenamyre, J. T., Hollingsworth, Z., Albin, R., D'Amato, C., Shoulson, I., Penney, J. B., 1988. NMDA receptor losses in putamen from patients with Huntington's disease. Science 241, 981-983.

Zhu, Y., Roth-Eichhorn, S., Braun, N., Culmsee, C., Rami, A., Krieglstein, J., 2000. The expression of transforming growth factor-beta1 (TGF-β1) in hippocampal neurons: a temporary upregulated protein level after transient forebrain ischemia in the rat. Brain Research Interactive 866, 286-298.